METHODS IN MOLECULAR BIOLOGY™

Series Editor
John M. Walker
School of Life Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For other titles published in this series, go to
www.springer.com/series/7651

Small Molecule Microarrays

Methods and Protocols

Edited by

Mahesh Uttamchandani

Defence Medical and Environmental Research Institute,
DSO National Laboratories, Singapore

Shao Q. Yao

Departments of Chemistry and Biological Sciences, Faculty of Science,
National University of Singapore, Singapore

Humana Press

Editors
Dr. Mahesh Uttamchandani
Defence Medical and Environmental
Research Institute
DSO National Laboratories
27 Medical Drive, #12-01,
Singapore 117510
mahesh@dso.org.sg

Dr. Shao Q.Yao
Department of Chemistry and Biological Sciences
Faculty of Science
National University of Singapore
Singapore
chmyaosq@nus.edu.sg

ISSN 1064-3745 e-ISSN 1940-6029
ISBN 978-1-60761-844-7 e-ISBN 978-1-60761-845-4
DOI 10.1007/978-1-60761-845-4

Library of Congress Control Number: 2010936183

Printed on acid-free paper

Humana Press is part of Springer Science+Business Media (www.springer.com)

Preface

Small Molecule Microarrays (SMMs): Methods and Protocols showcase a cornucopia of ways in which SMM technology may be deployed for multiplexed screening and profiling. SMMs were introduced just a decade ago in 1999 and, within a short space of time, have already established themselves as a vibrant, next generation platform for high-throughput screening. Significant developments, over the last decade, have equipped practitioners with an array of synthetic options, immobilization chemistries, assay modalities, and data processing schemes, thereby propelling the SMM platform to exciting new heights. Many of the scientists personally responsible for the success of these research endeavors have contributed chapters to this volume. As a result, the richness and diversity of this collection offers unique learning opportunities, both in fueling the development of new ideas and applications, as well as in tailoring proven solutions for different sets of libraries and microarray formats. Proven approaches, however, rarely work at the first attempt. Moreover, microarrays are notorious for the technical challenges they pose. It is to address these shortcomings that we have put together this volume, to supplement the open literature with practical tips for improved experimental design and the avoidance of potential pitfalls. The case studies in each chapter explain the key considerations and principles in getting things done right.

What challenges do working with SMMs pose? First and foremost is the complexity of the library synthesis and array fabrication steps. These processes lie at the core of SMM technology, for they govern how the molecules will be oriented and whether they will be accessible to the samples (such as proteins or proteomes) being screened. Quick and efficient means to construct pure and high-yielding libraries for SMMs is another major bottleneck; alternative approaches of array creation through in situ synthesis face similar challenges in ensuring the quality of the resulting arrays. The next major hurdle is in designing the methods for detecting positive readouts on the arrays and in using labels that minimally affect the interactions being probed. Finally, discerning between true interactions and false positives and improving signal to noise are among the challenges in enhancing readout quality and hit identification. These challenges are not without resolve. With careful preparation and informed decisions, these hurdles can be met and overcome using proven designs and strategies. Each chapter is designed to equip and inform the readers on these intricacies of SMM, as well as to provide the relevant guidance and means to harness and apply this technology.

The book is organized by the categories of small molecules presented on the microarrays. The molecules described herein include chemical libraries, peptide libraries, and carbohydrates. Each section covers a number of strategies for molecular immobilization, customized to the intended application. Chapter 1 provides an overview of SMM. Chapters 2–8 describe SMMs constructed from chemical libraries. A variety of covalent (Chaps. 2–3), noncovalent (Chaps. 4–5), and droplet-based techniques (Chaps. 6–7) are adopted for applications spanning enzyme profiling, inhibitor discovery, and in lysate protein screening (Chaps. 2–8). Chapters 9–15 describe peptide microarrays, including their fabrication using in situ synthesis (Chap. 9) and characterization with mass spectrometry (Chap. 10)

for applications in antibody detection (Chaps. 11–13) and kinase profiling (Chaps. 14–15). Chapters 16–17 describe ways in which carbohydrate microarrays may be fabricated (Chap. 16) and applied to glycobiology (Chaps. 16–17).

In reality, words alone are insufficient to learn the tricks of the microarray trade. The essential skills can only be learnt through hands-on experience and feedback at the bench. Each contributor has faced problems that have been solved with creativity and imagination. The chapters have been crafted to inspire a future generation of microarray practitioners to take the technology forward. In time, these individuals will create novel solutions of their own, which may possibly appear in future editions of the *Methods in Molecular Biology* series.

We thank the authors of each chapter for their willingness to share their expertise and insight. We are also grateful to John Walker for his invaluable advice and guidance and to the staff of Springer for taking the final steps to publication.

Singapore ***Mahesh Uttamchandani***
Singapore ***Shao Q. Yao***

Contents

Contributors

Olga Aminova • *Department of Chemistry and The Center for Excellence in Bioinformatics and Life Sciences, University at Buffalo, Buffalo, NY, USA*

Kang L. D. Aw • *Defence Medical and Environmental Research Institute, DSO National Laboratories, Singapore*

Ralf Bischoff • *Department of Chip-Based Peptide Libraries, German Cancer Research Center, Heidelberg, Germany*

Rabah Boukherroub • *Institut de Recherche Interdisciplinaire (IRI), CNRS-USR 3078,Villeneuve d'Ascq, France R. Boukherroub; Institut d'Electronique, de Microélectronique et de Nanotechnologie (IEMN), UMR CNRS 8520,Villeneuve d'Ascq, France*

Frank Breitling • *Institute for Microstructure Technology (IMT), Karlsruhe Institute of Technology (KIT), Eggenstein-Leopoldshafen, Germany*

Karim Chemlal • *INSERM U1019, Center for Infection and Immunity, CNRS UMR8204, IFR 142 Molecular and Cellular Medicine, Lille, France; INSERM U629, Institut Pasteur de Lille, Lille, France*

Yun-Chien Cheng • *Institute of Printing Science and Technology, Darmstadt University of Technology, Darmstadt, Germany*

Marcella Chiari • *Istituto di Chimica del Riconoscimento Molecolare (ICRM) C.N.R, Milano, Italy*

Marina Cretich • *Istituto di Chimica del Riconoscimento Molecolare (ICRM) C.N.R, Milano, Italy*

Francesco Damin • *Istituto di Chimica del Riconoscimento Molecolare (ICRM) C.N.R, Milano, Italy*

Anne-Sophie Debrie • *IINSERM U1019, Center for Infection and Immunity, CNRS UMR8204, IFR 142 Molecular and Cellular Medicine, Lille, France; INSERM U629, Institut Pasteur de Lille, Lille, France*

Rémi Desmet • *UMR 8161 CNRS-Université de Lille Nord de France, Lille, France; Institut Pasteur de Lille, Lille, France; IFR 142 Molecular and Cellular Medicine, Institut de Biologie de Lille, 1 rue de pr. calmette, 59021, Lille, France*

Scott L. Diamond • *Department of Chemical and Biomolecular Engineering, Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA, USA*

Eric Diesis • *UMR 8161 CNRS-Université de Lille Nord de France, Lille, France Institut Pasteur de Lille, Lille, France; IFR 142 Molecular and Cellular Medicine, Lille, France*

Matthew D. Disney • *Department of Chemistry and The Center for Excellence in Bioinformatics and Life Sciences, University at Buffalo, Buffalo, NY, USA*

Edgar Dörsam • *Institute of Printing Science and Technology, Darmstadt University of Technology, Darmstadt, Germany*

Hervé Drobecq • *UMR CNRS 8161, Université de Lille Nord de France, Institut Pasteur de Lille, IFR 142, Institut de Biologie de Lille, 1 rue du Pr. Calmette, 59021 Lille, France*
Gunter Fischer • *Max Planck Research Unit for Enzymology of Protein Folding, Halle, Germany*
Clelia Galati • *STMicroelectronics, Catania, Italy*
Dhaval Gosalia • *Department of Chemical and Biomolecular Engineering, Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA, USA*
Cecilia Gotti • *Istituto di Neuroscienze (IN) C.N.R, Milano, Italy*
Dipartimento di Farmacologia, Chemioterapia e Tossicologia Medica, Università degli Studi di Milano, Milano, Italy
Xiaoming Han • *Graduate School of System Life Sciences, Kyushu University, Fukuoka, Japan*
Jean-Michel Hougardy • *Laboratory of Vaccinology and Mucosal Immunology, Erasme Hospital, Université Libre de Bruxellers, 808, rooute de Lennik, B-1070 Brussels, Belgium*
Naoki Kanoh • *Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan*
Yoshiki Katayama • *Faculty of Engineering Graduate School of System Life Sciences and Department of Applied Chemistry, Center for Future Chemistry, Kyushu University, Fukuoka, Japan; CREST, JST, Japan*
Angela N. Koehler • *Broad Institute of Harvard and MIT, Cambridge MA, USA*
Kai König • *Department of Chip-Based Peptide Libraries, German Cancer Research Center, Heidelberg, Germany*
Hyang Yeon Lee • *Department of Chemistry, Seoul National University, Seoul, Korea*
Myung-Ryul Lee • *Department of Chemistry, Yonsei University, Seoul, Korea*
Camille Locht • *INSERM U1019, Center for Infection and Immunity, CNRS UMR8204, IFR 142 Molecular and Cellular Medicine, Lille, France; INSERM U629, Institut Pasteur de Lille, Lille, France*
Felix Löffler • *Department of Chip-Based Peptide Libraries, German Cancer Research Center, Heidelberg, Germany*
Renato Longhi • *Istituto di Chimica del Riconoscimento Molecolare (ICRM) C.N.R, Milano, Italy*
Frieder Märkle • *Department of Chip-Based Peptide Libraries, German Cancer Research Center, Heidelberg, Germany*
Françoise Mascart • *Laboratory of Vaccinology and Mucosal Immunology, Erasme Hospital, Université Libre de Bruxellers, 808, rooute de Lennik, B-1070 Brussels, Belgium*
Antonia Masch • *JPT Peptide Technologies GmbH, Berlin, Germany*
Oleg Melnyk • *UMR CNRS 8161, Université de Lille Nord de France, Institut Pasteur de Lille, IFR 142, Institut de Biologie de Lille, 1 rue du Pr. Calmette, 59021 Lille, France*
Isao Miyazaki • *Chemical Biology Department, RIKEN, Saitama, Japan*

Alexander Nesterov • *Institute for Microstructure Technology (IMT), Karlsruhe Institute of Technology (KIT), Eggenstein-Leopoldshafen, Germany*
Hiroyuki Osada • *Chemical Biology Department, RIKEN, Saitama, Japan*
Seung Bum Park • *Departments of Chemistry, Biophysics and Chemical Biology, Seoul National University, Seoul, Korea*
Sungjin Park • *Department of Chemistry, Yonsei University, Seoul, Korea*
Gaëlle Piret • *Institut de Recherche Interdisciplinaire (IRI), CNRS-USR 3078, Villeneuve d'Ascq, France; Institut d'Electronique, de Microélectronique et de Nanotechnologie (IEMN), UMR CNRS 8520, Villeneuve d'Ascq, France*
Ulf Reimer • *JPT Peptide Technologies GmbH, Berlin, Germany*
Lucio Renna • *STMicroelectronics, Catania, Italy*
Carine Rouanet • *INSERM U1019, Center for Infection and Immunity, CNRS UMR8204, IFR 142 Molecular and Cellular Medicine, Lille, France; INSERM U629, Institut Pasteur de Lille, Lille, France*
Christopher Schirwitz • *Department of Chip-Based Peptide Libraries, German Cancer Research Center, Heidelberg, Germany*
Mike Schutkowski • *JPT Peptide Technologies GmbH, Berlin, Germany; Institute of Biochemistry and Biotechnology, Martin Luther University Halle-Wittenberg, Halle, Germany*
Haibin Shi • *The NUS MedChem Program of the Office of Life Sciences, Faculty of Science, National University of Singapore, Singapore*
Injae Shin • *Department of Chemistry, Yonsei University, Seoul, Korea*
Siro Simizu • *Chemical Biology Department, RIKEN, Saitama, Japan*
Volker Stadler • *Department of Chip-Based Peptide Libraries, German Cancer Research Center, Heidelberg, Germany*
Alexandra Thiele • *Max Planck Research Unit for Enzymology of Protein Folding, Halle, Germany*
Mahesh Uttamchandani • *Defence Medical and Environmental Research Institute, DSO National Laboratories, Singapore; Deparments of Chemistry and Biological Sciences, National University of Singapore, Singapore*
Arturo J. Vegas • *Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge MA, USA; Koch Institute for Integrative Cancer Research, CambridgeMA, USA; Department of Anesthesiology, Children's Hospital Boston, Boston MA, USA*
Matthias Weiwad • *Max Planck Research Unit for Enzymology of Protein Folding, Halle, Germany*
Holger Wenschuh • *JPT Peptide Technologies GmbH, Berlin, Germany*
Shao Q. Yao • *Departments of Chemistry and Biological Sciences, and The NUS MedChem Program of the Office of Life Science, Faculty of Science, National University of Singapore, Singapore*
Johannes Zerweck • *JPT Peptide Technologies GmbH, Berlin, Germany*

Chapter 1

The Expanding World of Small Molecule Microarrays

Mahesh Uttamchandani and Shao Q. Yao

Abstract

Speed and throughput are vital ingredients for discovery-driven, "-omics" research. The small molecule microarray is one such platform, which delivers phenomenal screening throughput and capabilities. The concept at the heart of the technology is elegant, yet simple: by presenting large collections of molecules at a high density on a flat surface, one is able to interrogate them quickly and conveniently, evaluating all possible interactions in a single step. SMMs have, over the last decade, been established as a robust platform for screening, lead discovery, and molecular characterization. In this chapter, we describe the ways in which microarrays have been constructed and applied, focusing on the practical challenges faced when designing and performing SMM experiments. This is written as an introduction for new readers to the field, explaining the key principles and laying the foundation for the chapters that follow.

Key words: Small molecule microarrays, High-throughput screening, Combinatorial chemistry, Solid-phase synthesis, Proteomics, Glycomics, Ligand discovery, Enzyme-profiling, Drug discovery

1. Introduction

Small is big in the twenty-first century. Nowhere has this metaphor gained greater momentum than through the development and use of microarray technology (1, 2). Microarrays are miniaturized assemblies of molecules organized across a planar surface. The physical location of each spot on the array encodes its identity. Anywhere from the hundreds to tens of thousands of samples may be densely populated on planar surfaces, typically glass slides. The spectrum of applications is determined by the nature and class of molecules immobilized. Microarrays are hence categorized by the type of molecules presented. DNA microarrays, for instance, were developed in the mid 1990s by Brown and colleagues and comprise surfaces with addressed oligonucleotides (3, 4). Each spot on the array displays a known DNA sequence;

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_1, © Springer Science+Business Media, LLC 2010

thousands of them collectively on the microarray act like 'probes' to, quantitatively or comparatively, hybridize fluorescently labeled DNA from complex samples. This offers tremendous potential for applications in profiling the expression levels of mRNA and in identifying chromosomal abnormalities other genetic differences across samples (5–7). DNA microarrays continue to be widely applied and provide an unprecedented view into comparative genomics and genetics. They were the first in a long pipeline of a variety of different microarray types.

As the chemistries improved, a variety of molecules other than DNA, including proteins (8, 9), peptides (10), carbohydrates (11, 12), and chemical libraries (13, 14) were likewise arrayed and presented on microarrays. This happened around the turn of the century, when the Schreiber group developed microarrays containing small molecules in 1999 and proteins in 2000 (15, 16). With these exciting developments, it did not take long before proteome arrays (17), cell arrays (18), and tissue arrays (19) also emerged, all within the first quarter of the decade. The essence of what makes microarray technology so successful is its ability to miniaturize and parallelize assays (1, 20, 21). Microarrays of small molecules, the topic of this book, cover both synthetic and natural libraries of peptides, carbohydrates, and chemical compound libraries. Simply put, the application of these microarrays is biological screening; but covers specialized applications in protein fingerprinting, ligand discovery, and enzyme–substrate characterization. The rules by which immobilized small molecules interact with their targets are not as predictable as the rules of base pairing for DNA hybridization. Being able to assess these interactions in high-throughput thus offers valuable potential for drug discovery, discriminates proteins by their patterns of interactions, and provides insight into molecular interactions and structure-activity relationships (22). Furthermore, if many substrates are arrayed, it provides a window into identifying the most preferred substrate for any given enzyme (23, 24). These experiments are maturing to a point that they are not just being carried out with purified targets, but within whole proteomes or cellular lysates (25, 26), in order to interrogate protein–protein interactions and analyze the pathways involved in regulating protein function (23, 27, 28).

This book celebrates the first decade of small molecule microarrays. Over the last 10 years, there have been many surface chemistries and library synthesis schemes introduced to facilitate SMM creation. Commercial manufacturers are fabricating prespotted microarrays, allowing users to proceed directly with their experiments, without having to establish workflows and quality control for libraries and microarrays (29). We anticipate that the growing commercial support will better enable laboratories to embark on microarray projects, extending the spectrum of applications in the

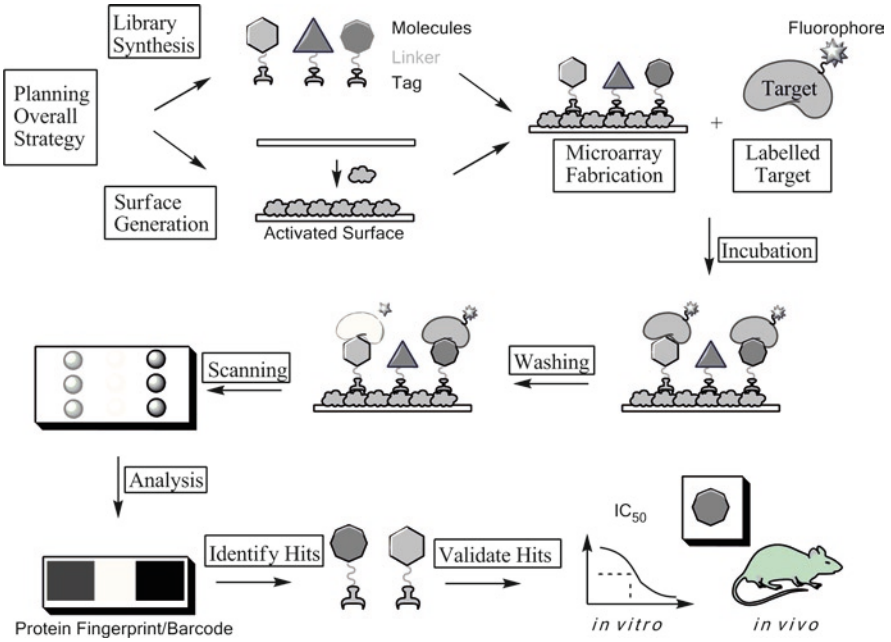


Fig. 1. Overview of SMM strategy applied to 'hit' identification for applications in drug discovery. Multiple proteins may also be screened using microarrays to provide individual protein barcodes or fingerprints.

years ahead. In this introductory chapter, we explain the fundamental concepts and challenges in constructing and applying this class of microarrays (Fig. 1). These considerations will be echoed throughout the methods in the book. The discussions here thus serve to guide a reader who may be unfamiliar with the design and application phases of SMMs.

2. Library Design and Synthesis

Combinatorial chemistry has provided the means to rapidly generate broad sets of compounds. The use of solid supports, split-pool synthesis, and encoding techniques (tea bag synthesis) have facilitated the synthesis of vast libraries of compounds, including peptides, drug-like molecules, and carbohydrates (8, 30). In the library design process, one must not only take into account a workflow that produces high yielding and sufficiently pure compounds (>80%) that would allow libraries to be arrayed directly without the need for purification, but also the incorporation of suitable tags and linkers (31). Synthesis on solid support (like polymer resins) provides a variety of advantages over solution phase synthesis. Through automation, repeated cycles of coupling can be easily carried out, and the use of such solid supports allows

reactions to be driven to completion (by using high concentrations of reactants), ensuring high-yields and purities for standard, well-optimized chemistries. Reactions that give low yields are, however, not amenable to synthesis on solid support, as iterations on solid support will diminish the overall yield and purity. Synthetic strategies on solid support include position scanning libraries, alanine-scanning libraries, diversity-oriented synthesis, and other approaches (13, 32, 33). Other strategies for library synthesis include fragment-based approaches and strategies like click-chemistry that have been applied to create diverse collections of molecules for microarray applications (22).

It is desirable for the identity of each molecule spotted be known a priori, before the arraying process. This, however, imposes a considerable burden to the synthesis, as it comes at the expense of added time, effort, and cost. Certain groups have sought to use mass spectrometry to deconvolute identified hits after sample application instead (13, 33). Tea bag style synthesis, on the other hand using radiofrequency tags, has made it possible to synthesize large chemical libraries, with each member identifiable at the end of the synthesis (34). In the earlier years, the investment in expensive equipment and reactors for combinatorial synthesis was one barrier limiting access to the technology. Over the last decade, commercial synthetic labs have been built, and even those within core facilities at universities now have the means to offer simple peptide library synthesis services. This could in future be applied to the synthesis of more complex small molecules for microarray construction. Prefabricated arrays and coated microarrays slides have also become available from commercial vendors (29).

The synthesis process must at some point, include a tag to enable covalent immobilization on the arrays (31). If the tag is generic (like a common amine or carboxylic acid or aldehyde) and appears at multiple locations of the molecules, it results in regio-specific immobilization. In this context, any of the tags present within the molecule could bind to the functionalized microarray surface, hence presenting the molecules in one of several possible orientations. This results in a mixed orientation of molecules within a spot. The pH of the immobilization buffer used can, for example, favor immobilization of terminal amines in place of epsilon amines of lysines because of their different acid dissociation constants (pKa).

Site-specific immobilization involves a unique tag present at one predefined position in the library. This tag chemoselectively binds the functionalized array surface. For most applications, site-specific immobilization is preferred as the molecules are homogenously presented on the array surface. Where the binding epitopes are unknown, or where one would like to present more facets of the molecules for interaction, regio-specific immobilization may

be preferred, to not constrain the molecules to any predefined orientation (35, 36). Certain tags that facilitate non-covalent, but nevertheless stable immobilization of molecules onto the surface may also be applied. These include the use of polyhistidine tag (with a nickel or NTA surface) (37), the biotin tag (with an avidin surface) (38), and epitope tag (like flag or glutathione-S-transferase, GST) for immobilization on antibody-coated surfaces (17, 39). In certain cases, peptide nucleic acids or other oligonucleotides have also been used as tags for small molecule libraries, where positive molecular 'hits' can be identified through hybridization onto DNA microarrays (40–42).

3. Array Fabrication

Using high precision robotic printers, one is able to automatically deposit libraries of compounds from 384-well plates onto pre-coated glass slides. These are the same commercial spotters used for DNA microarray fabrication, and create spots by contact printing (using microarray pins) or piezoelectric deposition. As the same pins are recycled for different samples, it is important to ensure proper washing of pins in solvents like water or ethanol between sample applications. For such spotted arrays, this allows around 5,000 molecules to be printed in duplicate on a standard glass microscope slide, with each spot typically having a diameter of 80–200 μm. Alternatively, arrays may be built in situ, where chains of peptides and similar polymers may be sequentially grown on the surface itself, akin to building high rise apartment blocks, level by level (43, 44). For such in situ synthesized chips, the array density can be a lot greater, with feature dimensions going down to just several microns, allowing more than a million features to fit on the area the size of a thumbnail (45, 46).

Immobilizing molecules stably onto chips is the most fundamental step during microarray construction. Factors such as molecular orientation, immobilization chemistry, and stability are key considerations that govern downstream application (Fig. 2). A variety of "capture" agents such as aptamers, antibodies, and chemical tags are available to immobilize small molecules on microarrays. These strategies have been reviewed extensively elsewhere (8, 21, 31, 47, 48). To illustrate several notable examples, groups have developed orthogonal tag systems, where highly specific reactions may be used for immobilization of libraries on arrays. This includes the use of click-chemistry type ligations, including the Staudinger ligation between a phosphane and azide moiety (49) and the 1–3 dipolar cycloaddition reaction between and alkynes and azides (50). The hydrazide surface has also been applied in the immobilization of carbohydrates and other biomolecules on SMMs (51).

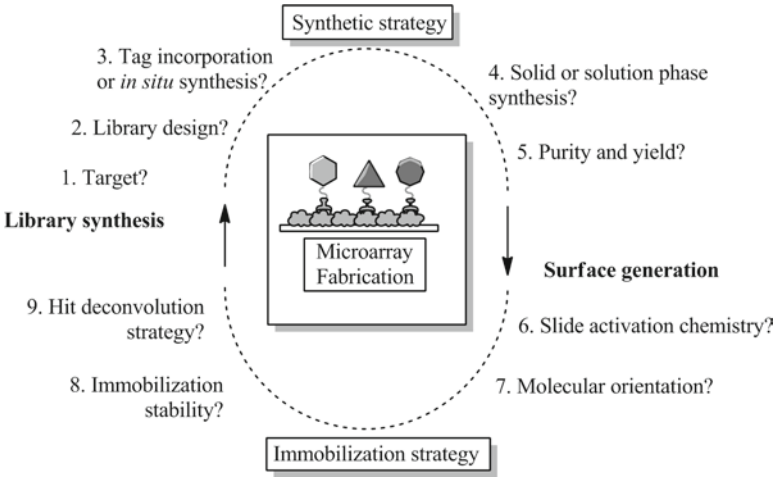


Fig. 2. Key points during the early stages of microarray construction. These usually involve interdependent considerations in library design and array fabrication.

Separately, there have been demonstrations on the use of inteins, which are short proteins capable of self-splice for tagging and ligating molecules onto arrays (38, 52).

Photoactivation chemistries have alternatively been applied for the controlled activation of surfaces for chemical ligation (53, 54) (Fig. 3). These chemistries have applications both in the iterative synthesis of molecules in situ, as well as in the capture of spotted samples. The capture of molecules in this manner may be site-specific where complementary tags are incorporated in the library members or nonspecific where such tags are not required. The latter approach has the advantage of being able to immobilize molecules without the need of tags, allowing natural extracts or compounds to be presented on microarrays for simultaneous analysis (54). Other notable examples have facilitated in situ assembly of molecules in high density on microarrays have utilized light-directed synthesis, with or without photomasks (55, 56), photogenerated acids (57), or precision delivery of activated-amino acids in particles using electric fields (44) (Fig. 3). These have the advantage of building microarrays from the surface up. Like any other solid phase methodology, they require that every coupling step be highly efficient and robust, to ensure high quality of the arrays. They are similarly limited by the length of the features that can be synthesized, as every coupling cycle diminishes the theoretical yield and purity attainable. The complexity is also greater in fabricating peptides because of 20 possible amino acid combinations at every layer, compared to just four building blocks for nucleotides. This method is applied commercially for the synthesis of DNA microarrays, but with promising recent

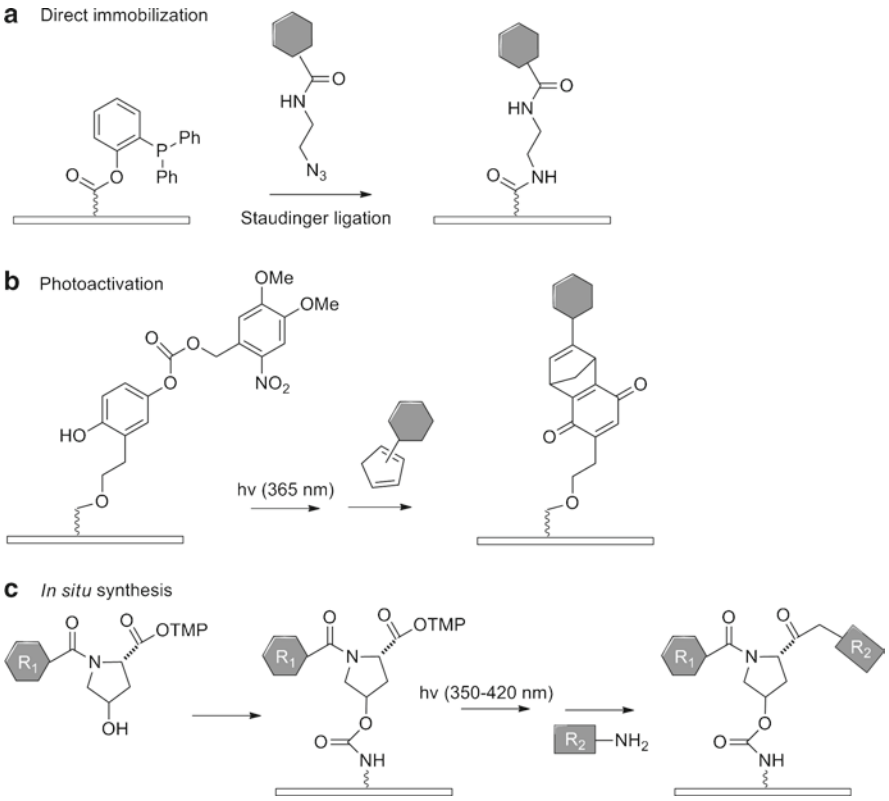


Fig. 3. Various strategies for fabricating SMMs. (**a**) Covalent, site-specific immobilization using Staudinger ligation. The molecules are tagged with azide, and the slide surface is coated with phosphane (49). (**b**) Hydroxyquinone groups on gold substrates that were protected with nitroveratryoxycarbonyl, a photolabile group. Molecules were tagged with cyclopentadienes (53). (**c**) Sequential molecular assembly in situ. The first set of building blocks is tagged with photolabile trimethyoxyphenacyl group, and the second set with amine (56).

developments (44), could also be applied cost-effectively for peptide and other types of small molecule microarray synthesis.

An alternative method of presentation on SMMs has been the application of small molecules in droplets (58). This takes the SMM concept to another dimension as it overcomes the need of immobilizing both the molecules and targets allowing reactions to be performed in solution phase, in a label-free manner. This has facilitated the screening of proteases and other enzymes using such nanodroplet microarrays (24, 59, 60) (Fig. 4a). The use of glycerol and incubations in humid environments prevents the spots from drying up during the incubation steps. A similar application was in encapsulating small molecules within biodegradable polymers on arrays, upon which layers of cells were grown (61). The hydrolysis of the polymer capsule and release of the molecules facilitated high-throughput screening of small molecules, and facilitated assessments on their effects on cell growth.

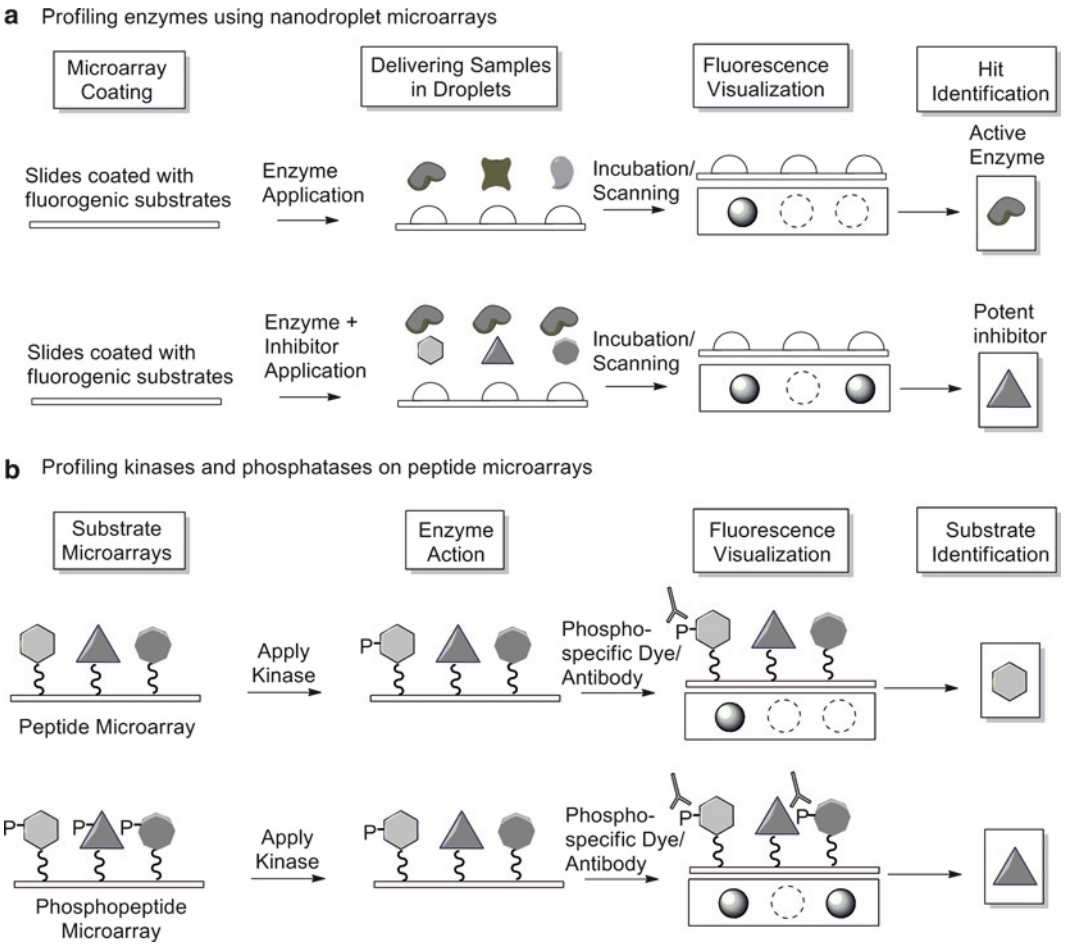


Fig. 4. Applications of SMMs in enzyme profiling and inhibitor discovery. (**a**) The use of droplet microarrays for screening enzymes (*top*) and inhibitors (*bottom*) (59, 82, 89). (**b**) Methods for screening kinases (*top*) (74–76) and phosphatases (*bottom*) (77, 78) using peptide microarrays.

4. Applications of SMMs

Biological evaluation of small molecules is an important first step in the drug discovery process. SMMs provide a valuable opportunity to screen drug targets to assess molecular interactions in high-throughput. One of the key requirements of this process is the ability to visualize the positive interactions, which is usually facilitated by directly tagging the protein of interest with a fluorophore (Fig. 1). This provides a direct fluorescent readout to the protein that remains bound to locations on the array following the wash steps, indicating the molecules on the array to which it most tightly binds. The tags used typically are cyanine dyes (e.g., Cy3/Cy5) or fluorescein dyes that are available commercially as active esters. These dyes, under basic conditions, can be bound

minimally to target proteins through conjugation with their terminal amines. Alternatively expressing proteins with fluorescence protein tags (like GFP) is an alternative method of producing 'visible' proteins. However, for proteins where the N-terminus needs to be free to preserve function, or where labeling detriments the activity of the protein of interest, the alternative visualization approaches should be used.

Antibody-based methods provide one such alternative. Antibodies for many proteins and biological targets are available commercially in either polyclonal or monoclonal forms. In this scenario, the protein is first applied, in an unlabeled form, to the microarrays. Following mild or harsh washing cycles, as may be desired to improve signal to noise, antibodies labeled with fluorophores, are applied to detect the proteins on the microarrays. A cautionary note when using such an approach is to screen the microarrays just with the antibody first (without the target) to ensure that the antibody does not directly bind features on the array. If significant binding is observed across the array, an alternative detection system should be used, or switch to a monoclonal antibody variant instead which could afford greater specificity to the protein target. If minimal background is observed, these readouts could be subtracted from results that run in parallel with the protein present (or even ignored if insignificant). Sometimes, target proteins have tags or epitopes available that were used for affinity purification. Antibodies against these tags (like anti-GST, anti-MBP, or anti-His) are also available and may also be applied to detect the proteins on the microarrays.

Mass-spectrometry represents another platform that has been applied in tandem with microarrays, both in the isolation of molecules for array fabrication (62), as well as the detection of positive readouts on the microarray (63), thus moving away from the need for fluorescent-based detection. Surface plasmon resonance and imaging methods (like interferometry) have also been developed to facilitate the detection of readouts in high-throughput on microarrays, without having to use tags (64–67).

The other challenge posed by SMMs is the analysis of the wealth of data obtained (Fig. 1). Usually, the brightest spots are identified and pulled out for further analysis. The number of molecules that represent ultimately depends on the ability to validate the hits, according to the thresholds set for the experiment. Inevitably, many of the initial hits may not bind the appropriate binding pocket, or may turn out not to have the desired biological effects on the target. The microarray should thus be seen as the first step in the screening process to identify molecules with possible ligand binding potential; the hits identified should be further validated in specific and more quantitative assays for the target to test for the actual binding/inhibition potency using K_D, K_i, or IC_{50} measurements (13, 33, 68, 69).

Methodologies have been developed through controlled time or concentration-dependent applications to derive dissociation and inhibition constants directly from the microarray readouts themselves (70, 71). Another option is to develop substrate arrays in place of ligand arrays; the readouts obtained upon enzyme application more directly indicate the actual activity of the proteins (72, 73). Accordingly, a wide variety of substrate microarrays have been applied for assessing the activity of enzymes, such as kinases (25, 74–76), phosphatases (23, 77, 78), transferases (79–81), and proteases (66, 82, 83) (Fig. 4b). Alternatively, activity-based probes have also facilitated the assessment of enzymatic activity on microarrays (70, 84).

Besides assessments of enzymatic activity, SMMs have also been applied to protein fingerprinting (85–88). This is in a way a byproduct of microarrays, as every sample applied produces a fingerprint. However, looking at these signatures comparatively, offers tremendous insight into the functional differences especially across a class of closely related proteins. This is valuable, more so, because it comes from the perspective of thousands of interacting small molecule ligands, and has the added advantage of being able to identify "hits" on the microarray that are selective to the desired target of interest, without binding its closely related partners.

5. Conclusion

Here, we have discussed a variety of practical issues and challenges involved in the fabrication and application of SMMs. These include various points in the design of libraries for immobilization onto SMMs, the need for tags both on the small molecules (for immobilization), and targets of choice (for visualization). Careful preparation is needed to consider all the experimental steps and options in order to draw up a successful SMM workflow. The quality of the libraries and reproducibility of the resulting microarrays is of utmost importance. The preservation of the protein activity during the sample application phase is also critical to ensure good quality results.

As SMM technology matures and evolves, a great deal of insight will be gained on the targets screened and the pathways they control. Through the development of databases of molecular interactions, platforms such as SMMs will contribute to our knowledge of how small molecules behave and interact with biological targets within complex living systems. Perhaps, such knowledge will one day enable us to predict the best possible ligand for any given protein, without having to perform a single experiment.

Further developments in microfluidics could also take advantage of the throughput offered by SMMs, for applications in detection and diagnostics.

The first decade of SMMs has been an exciting one, with much interest in new applications and fabrication strategies. Now, the technology has matured greatly, with a wider spectrum of laboratories becoming equipped to deploy the platform for their collection of chemical libraries or biological targets. Drug companies would also perhaps in the next decade begin applying SMMs for routine, cost-effective screening, taking over from classical workflows using microplates. A next generation of 'nano'arrays, using nanolithography and other techniques, is also being developed, which aims to reduce the feature sizes on arrays by several orders of magnitude. With these exciting developments, the next decade promises even more fantastic breakthroughs and discoveries using SMMs.

Acknowledgments

We gratefully acknowledge financial support by MOE (R143-000-394-112), BMRC (R143-000-391-305), CRP (R143-000-218-281), and DSO National Laboratories.

References

1. Hu, Y., Uttamchandani, M., and Yao, S. Q. (2006) Microarray: a versatile platform for high-throughput functional proteomics, *Comb Chem High Throughput Screen 9*, 203–212.
2. MacBeath, G., and Saghatelian, A. (2009) The promise and challenge of '-omic' approaches, *Curr Opin Chem Biol 13*, 501–502.
3. Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray, *Science 270*, 467–470.
4. Schena, M., Shalon, D., Heller, R., Chai, A., Brown, P. O., and Davis, R. W. (1996) Parallel human genome analysis: microarray-based expression monitoring of 1000 genes, *Proc Natl Acad Sci U S A 93*, 10614–10619.
5. Uttamchandani, M., Neo, J. L., Ong, B. N., and Moochhala, S. (2009) Applications of microarrays in pathogen detection and biodefence, *Trends Biotechnol 27*, 53–61.
6. Khan, J., Bittner, M. L., Chen, Y., Meltzer, P. S., and Trent, J. M. (1999) DNA microarray technology: the anticipated impact on the study of human disease, *Biochim Biophys Acta 1423*, M17–M28.
7. Yoo, S. M., Choi, J. H., Lee, S. Y., and Yoo, N. C. (2009) Applications of DNA microarray in disease diagnostics, *J Microbiol Biotechnol 19*, 635–646.
8. Uttamchandani, M., Wang, J., and Yao, S. Q. (2006) Protein and small molecule microarrays: powerful tools for high-throughput proteomics, *Mol Biosyst 2*, 58–68.
9. Templin, M. F., Stoll, D., Schrenk, M., Traub, P. C., Vohringer, C. F., and Joos, T. O. (2002) Protein microarray technology, *Trends Biotechnol 20*, 160–166.
10. Falsey, J. R., Renil, M., Park, S., Li, S., and Lam, K. S. (2001) Peptide and small molecule microarray for high throughput cell adhesion and functional assays, *Bioconjug Chem 12*, 346–353.
11. Park, S., and Shin, I. (2002) Fabrication of carbohydrate chips for studying protein-carbohydrate interactions, *Angew Chem Int Ed Engl 41*, 3180–3182.

12. Park, S., Lee, M. R., Pyo, S. J., and Shin, I. (2004) Carbohydrate chips for studying high-throughput carbohydrate-protein interactions, *J Am Chem Soc 126*, 4812–4819.
13. Kuruvilla, F. G., Shamji, A. F., Sternson, S. M., Hergenrother, P. J., and Schreiber, S. L. (2002) Dissecting glucose signalling with diversity-oriented synthesis and small-molecule microarrays, *Nature 416*, 653–657.
14. Hergenrother, P. J., Depew, K. M., and Schreiber, S. L. (2000) Small-molecule microarrays: covalent attachment and screening of alcohol-containing small molecules on glass slides, *J Am Chem Soc 122*, 7849–7850.
15. MacBeath, G., Koehler, A. N., and Schreiber, S. L. (1999) Printing small molecules as microarrays and detecting protein ligand interactions en masse, *J Am Chem Soc 121*, 7967–7968.
16. MacBeath, G., and Schreiber, S. L. (2000) Printing proteins as microarrays for high-throughput function determination, *Science 289*, 1760–1763.
17. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R. A., Gerstein, M., and Snyder, M. (2001) Global analysis of protein activities using proteome chips, *Science 293*, 2101–2105.
18. Ziauddin, J., and Sabatini, D. M. (2001) Microarrays of cells expressing defined cDNAs, *Nature 411*, 107–110.
19. Horvath, L., and Henshall, S. (2001) The application of tissue microarrays to cancer research, *Pathology 33*, 125–129.
20. Sun, H., Chattopadhaya, S., Wang, J., and Yao, S. Q. (2006) Recent developments in microarray-based enzyme assays: from functional annotation to substrate/inhibitor fingerprinting, *Anal Bioanal Chem 386*, 416–426.
21. Uttamchandani, M., Walsh, D. P., Yao, S. Q., and Chang, Y. T. (2005) Small molecule microarrays: recent advances and applications, *Curr Opin Chem Biol 9*, 4–13.
22. Uttamchandani, M., Lu, C. H., and Yao, S. Q. (2009) Next generation chemical proteomic tools for rapid enzyme profiling, *Acc Chem Res 42*, 1183–1192.
23. Sun, H., Lu, C. H., Uttamchandani, M., Xia, Y., Liou, Y. C., and Yao, S. Q. (2008) Peptide microarray for high-throughput determination of phosphatase specificity and biology, *Angew Chem Int Ed Engl 47*, 1698–1702.
24. Gosalia, D. N., Salisbury, C. M., Ellman, J. A., and Diamond, S. L. (2005) High throughput substrate specificity profiling of serine and cysteine proteases using solution-phase fluorogenic peptide microarrays, *Mol Cell Proteomics 4*, 626–636.
25. Shigaki, S., Yamaji, T., Han, X., Yamanouchi, G., Sonoda, T., Okitsu, O., Mori, T., Niidome, T., and Katayama, Y. (2007) A peptide microarray for the detection of protein kinase activity in cell lysate, *Anal Sci 23*, 271–275.
26. Evans, D., Johnson, S., Laurenson, S., Davies, A. G., Ko Ferrigno, P., and Walti, C. (2008) Electrical protein detection in cell lysates using high-density peptide-aptamer microarrays, *J Biol 7*, 3.
27. Stiffler, M. A., Grantcharova, V. P., Sevecka, M., and MacBeath, G. (2006) Uncovering quantitative protein interaction networks for mouse PDZ domains using protein microarrays, *J Am Chem Soc 128*, 5913–5922.
28. Jones, R. B., Gordus, A., Krall, J. A., and MacBeath, G. (2006) A quantitative protein interaction network for the ErbB receptors using protein microarrays, *Nature 439*, 168–174.
29. Eisenstein, M. (2006) Protein arrays: growing pains, *Nature 444*, 959–962.
30. Lam, K. S., and Renil, M. (2002) From combinatorial chemistry to chemical microarray, *Curr Opin Chem Biol 6*, 353–358.
31. Wang, J., Uttamchandani, M., Sun, H., and Yao, S. Q. (2006) Small molecule microarrays: applications using specially tagged chemical libraries. *QSAR Comb Sci 25*, 1009–1019.
32. Lee, A., and Breitenbucher, J. G. (2003) The impact of combinatorial chemistry on drug discovery, *Curr Opin Drug Discov Devel 6*, 494–508.
33. Koehler, A. N., Shamji, A. F., and Schreiber, S. L. (2003) Discovery of an inhibitor of a transcription factor using small molecule microarrays and diversity-oriented synthesis, *J Am Chem Soc 125*, 8420–8421.
34. Xiao, X. Y., Li, R., Zhuang, H., Ewing, B., Karunaratne, K., Lillig, J., Brown, R., and Nicolaou, K. C. (2000) Solid-phase combinatorial synthesis using MicroKan reactors, Rf tagging, and directed sorting. *Biotechnol Bioeng 71*, 44–50.
35. Kanoh, N., Asami, A., Kawatani, M., Honda, K., Kumashiro, S., Takayama, H., Simizu, S., Amemiya, T., Kondoh, Y., Hatakeyama, S., Tsuganezawa, K., Utata, R., Tanaka, A., Yokoyama, S., Tashiro, H., and Osada, H. (2006) Photo-cross-linked small-molecule microarrays as chemical genomic tools for dissecting protein-ligand interactions, *Chem Asian J 1*, 789–797.

36. Kanoh, N., Takayama, H., Honda, K., Moriya, T., Teruya, T., Simizu, S., Osada, H., and Iwabuchi, Y. (2010) Cleavable linker for photocross-linked small-molecule affinity matrix. *Bioconjug Chem 21*, 182–186.
37. Kwon, K., Grose, C., Pieper, R., Pandya, G. A., Fleischmann, R. D., and Peterson, S. N. (2009) High quality protein microarray using in situ protein purification. *BMC Biotechnol 9*, 72.
38. Lesaicherre, M. L., Uttamchandani, M., Chen, G. Y., and Yao, S. Q. (2002) Developing site-specific immobilization strategies of peptides in a microarray. *Bioorg Med Chem Lett 12*, 2079–2083.
39. Ramachandran, N., Raphael, J. V., Hainsworth, E., Demirkan, G., Fuentes, M. G., Rolfs, A., Hu, Y., and LaBaer, J. (2008) Next-generation high-density self-assembling functional protein arrays. *Nat Methods 5*, 535–538.
40. Hu, Y., Chen, G. Y., and Yao, S. Q. (2005) Activity-based high-throughput screening of enzymes by using a DNA microarray. *Angew Chem Int Ed Engl 44*, 1048–1053.
41. Winssinger, N., and Harris, J. L. (2005) Microarray-based functional protein profiling using peptide nucleic acid-encoded libraries, *Expert Rev Proteomics 2*, 937–947.
42. Melkko, S., Scheuermann, J., Dumelin, C. E., and Neri, D. (2004) Encoded self-assembling chemical libraries, *Nat Biotechnol 22*, 568–574.
43. Gao, X., Pellois, J. P., Na, Y., Kim, Y., Gulari, E., and Zhou, X. (2004) High density peptide microarrays. In situ synthesis and applications, *Mol Divers 8*, 177–187.
44. Beyer, M., Nesterov, A., Block, I., Konig, K., Felgenhauer, T., Fernandez, S., Leibe, K., Torralba, G., Hausmann, M., Trunk, U., Lindenstruth, V., Bischoff, F. R., Stadler, V., and Breitling, F. (2007) Combinatorial synthesis of peptide arrays onto a microchip, *Science 318*, 1888.
45. Fodor, S. P., Rava, R. P., Huang, X. C., Pease, A. C., Holmes, C. P., and Adams, C. L. (1993) Multiplexed biochemical assays with biological chips, *Nature 364*, 555–556.
46. Breitling, F., Nesterov, A., Stadler, V., Felgenhauer, T., and Bischoff, F. R. (2009) High-density peptide arrays, *Mol Biosyst 5*, 224–234.
47. Duffner, J. L., Clemons, P. A., and Koehler, A. N. (2007) A pipeline for ligand discovery using small-molecule microarrays, *Curr Opin Chem Biol 11*, 74–82.
48. Pilobello, K. T., and Mahal, L. K. (2007) Deciphering the glycocode: the complexity and analytical challenge of glycomics, *Curr Opin Chem Biol 11*, 300–305.
49. Kohn, M., Wacker, R., Peters, C., Schroder, H., Soulere, L., Breinbauer, R., Niemeyer, C. M., and Waldmann, H. (2003) Staudinger ligation: a new immobilization strategy for the preparation of small-molecule arrays, *Angew Chem Int Ed Engl 42*, 5830–5834.
50. Lin, P. C., Ueng, S. H., Tseng, M. C., Ko, J. L., Huang, K. T., Yu, S. C., Adak, A. K., Chen, Y. J., and Lin, C. C. (2006) Site-specific protein modification through Cu(I)-catalyzed 1,2,3-triazole formation and its implementation in protein microarray fabrication, *Angew Chem Int Ed Engl 45*, 4286–4290.
51. Lee, M. R., and Shin, I. (2005) Facile preparation of carbohydrate microarrays by site-specific, covalent immobilization of unmodified carbohydrates on hydrazide-coated glass slides, *Org Lett 7*, 4269–4272.
52. Camarero, J. A., Kwon, Y., and Coleman, M. A. (2004) Chemoselective attachment of biologically active proteins to surfaces by expressed protein ligation and its application for "protein chip" fabrication, *J Am Chem Soc 126*, 14730–14731.
53. Dillmore, W. S., Yousaf, M. N., and Mrksich, M. (2004) A photochemical method for patterning the immobilization of ligands and cells to self-assembled monolayers, *Langmuir 20*, 7223–7231.
54. Kanoh, N., Kumashiro, S., Simizu, S., Kondoh, Y., Hatakeyama, S., Tashiro, H., and Osada, H. (2003) Immobilization of natural products on glass slides by using a photoaffinity reaction and the detection of protein-small-molecule interactions, *Angew Chem Int Ed Engl 42*, 5584–5587.
55. Fodor, S. P., Read, J. L., Pirrung, M. C., Stryer, L., Lu, A. T., and Solas, D. (1991) Light-directed, spatially addressable parallel chemical synthesis, *Science 251*, 767–773.
56. Shaginian, A., Patel, M., Li, M. H., Flickinger, S. T., Kim, C., Cerrina, F., and Belshaw, P. J. (2004) Light-directed radial combinatorial chemistry: orthogonal safety-catch protecting groups for the synthesis of small molecule microarrays, *J Am Chem Soc 126*, 16704–16705.
57. Pellois, J. P., Zhou, X., Srivannavit, O., Zhou, T., Gulari, E., and Gao, X. (2002) Individually addressable parallel peptide synthesis on microchips, *Nat Biotechnol 20*, 922–926.
58. Diamond, S. L. (2007) Methods for mapping protease specificity, *Curr Opin Chem Biol 11*, 46–51.
59. Gosalia, D. N., Salisbury, C. M., Maly, D. J., Ellman, J. A., and Diamond, S. L. (2005)

Profiling serine protease substrate specificity with solution phase fluorogenic peptide microarrays, *Proteomics 5*, 1292–1298.

60. Angenendt, P., Lehrach, H., Kreutzberger, J., and Glokler, J. (2005) Subnanoliter enzymatic assays on microarrays, *Proteomics 5*, 420–425.
61. Bailey, S. N., Sabatini, D. M., and Stockwell, B. R. (2004) Microarrays of small molecules embedded in biodegradable polymers for use in mammalian cell-based screens, *Proc Natl Acad Sci U S A 101*, 16144–16149.
62. Blake, T. A., Ouyang, Z., Wiseman, J. M., Takats, Z., Guymon, A. J., Kothari, S., and Cooks, R. G. (2004) Preparative linear ion trap mass spectrometer for separation and collection of purified proteins and peptides in arrays using ion soft landing, *Anal Chem 76*, 6293–6305.
63. Yu, X., Xu, D., and Cheng, Q. (2006) Label-free detection methods for protein microarrays, *Proteomics 6*, 5493–5503.
64. Lausted, C., Hu, Z., Hood, L., and Campbell, C. T. (2009) SPR imaging for high throughput, label-free interaction analysis, *Comb Chem High Throughput Screen 12*, 741–751.
65. Souplet, V., Desmet, R., and Melnyk, O. (2007) Imaging of protein layers with an optical microscope for the characterization of peptide microarrays, *J Pept Sci 13*, 451–457.
66. Inoue, Y., Mori, T., Yamanouchi, G., Han, X., Sonoda, T., Niidome, T., and Katayama, Y. (2008) Surface plasmon resonance imaging measurements of caspase reactions on peptide microarrays, *Anal Biochem 375*, 147–149.
67. Ozkumur, E., Needham, J. W., Bergstein, D. A., Gonzalez, R., Cabodi, M., Gershoni, J. M., Goldberg, B. B., and Unlu, M. S. (2008) Label-free and dynamic detection of biomolecular interactions for high-throughput microarray applications, *Proc Natl Acad Sci U S A 105*, 7988–7992.
68. Uttamchandani, M., Walsh, D. P., Khersonsky, S. M., Huang, X., Yao, S. Q., and Chang, Y. T. (2004) Microarrays of tagged combinatorial triazine libraries in the discovery of small-molecule ligands of human IgG, *J Comb Chem 6*, 862–868.
69. Disney, M. D., and Seeberger, P. H. (2004) The use of carbohydrate microarrays to study carbohydrate-cell interactions and to detect pathogens, *Chem Biol 11*, 1701–1707.
70. Funeriu, D. P., Eppinger, J., Denizot, L., Miyake, M., and Miyake, J. (2005) Enzyme family-specific and activity-based screening of chemical libraries using enzyme microarrays, *Nat Biotechnol 23*, 622–627.
71. Gordus, A., and MacBeath, G. (2006) Circumventing the problems caused by protein diversity in microarrays: implications for protein interaction networks, *J Am Chem Soc 128*, 13668–13669.
72. Zhu, Q., Uttamchandani, M., Li, D., Lesaicherre, M. L., and Yao, S. Q. (2003) Enzymatic profiling system in a small-molecule microarray, *Org Lett 5*, 1257–1260.
73. Salisbury, C. M., Maly, D. J., and Ellman, J. A. (2002) Peptide microarrays for the determination of protease substrate specificity, *J Am Chem Soc 124*, 14868–14870.
74. Uttamchandani, M., Chen, G. Y., Lesaicherre, M. L., and Yao, S. Q. (2004) Site-specific peptide immobilization strategies for the rapid detection of kinase activity on microarrays, *Methods Mol Biol 264*, 191–204.
75. Stoevesandt, O., Elbs, M., Kohler, K., Lellouch, A. C., Fischer, R., Andre, T., and Brock, R. (2005) Peptide microarrays for the detection of molecular interactions in cellular signal transduction, *Proteomics 5*, 2010–2017.
76. Rychlewski, L., Kschischo, M., Dong, L., Schutkowski, M., and Reimer, U. (2004) Target specificity analysis of the Abl kinase using peptide microarray data, *J Mol Biol 336*, 307–311.
77. Sun, H., Tan, L. P., Gao, L., and Yao, S. Q. (2009) High-throughput screening of catalytically inactive mutants of protein tyrosine phosphatases (PTPs) in a phosphopeptide microarray, *Chem Commun (Camb)*, 677–679.
78. Kohn, M., Gutierrez-Rodriguez, M., Jonkheijm, P., Wetzel, S., Wacker, R., Schroeder, H., Prinz, H., Niemeyer, C. M., Breinbauer, R., Szedlacsek, S. E., and Waldmann, H. (2007) A microarray strategy for mapping the substrate specificity of protein tyrosine phosphatase, *Angew Chem Int Ed Engl 46*, 7700–7703.
79. Park, S., and Shin, I. (2007) Carbohydrate microarrays for assaying galactosyltransferase activity, *Org Lett 9*, 1675–1678.
80. Bryan, M. C., Lee, L. V., and Wong, C. H. (2004) High-throughput identification of fucosyltransferase inhibitors using carbohydrate microarrays, *Bioorg Med Chem Lett 14*, 3185–3188.
81. Ban, L., and Mrksich, M. (2008) On-chip synthesis and label-free assays of oligosaccharide arrays, *Angew Chem Int Ed Engl 47*, 3396–3399.
82. Wang, J., Uttamchandani, M., Sun, L. P., and Yao, S. Q. (2006) Activity-based high-throughput profiling of metalloprotease inhibitors

using small molecule microarrays, *Chem Commun (Camb)*, 717–719.

83. Scheuermann, J., Dumelin, C. E., Melkko, S., Zhang, Y., Mannocci, L., Jaggi, M., Sobek, J., and Neri, D. (2008) DNA-encoded chemical libraries for the discovery of MMP-3 inhibitors, *Bioconjug Chem 19*, 778–785.
84. Chen, G. Y., Uttamchandani, M., Zhu, Q., Wang, G., and Yao, S. Q. (2003) Developing a strategy for activity-based detection of enzymes in a protein microarray, *Chembiochem 4*, 336–339.
85. Reddy, M. M., and Kodadek, T. (2005) Protein "fingerprinting" in complex mixtures with peptoid microarrays, *Proc Natl Acad Sci U S A 102*, 12672–12677.
86. Usui, K., Ojima, T., Takahashi, M., Nokihara, K., and Mihara, H. (2004) Peptide arrays with designed secondary structures for protein characterization using fluorescent fingerprint patterns, *Biopolymers 76*, 129–139.
87. Usui, K., Tomizaki, K. Y., and Mihara, H. (2006) Protein-fingerprint data mining of a designed alpha-helical peptide array, *Mol Biosyst 2*, 417–420.
88. Uttamchandani, M., Wang, J., Li, J., Hu, M., Sun, H., Chen, K. Y., Liu, K., and Yao, S. Q. (2007) Inhibitor fingerprinting of matrix metalloproteases using a combinatorial peptide hydroxamate library, *J Am Chem Soc 129*, 7848–7858.
89. Uttamchandani, M., Huang, X., Chen, G. Y., and Yao, S. Q. (2005) Nanodroplet profiling of enzymatic activities in a microarray, *Bioorg Med Chem Lett 15*, 2135–2139.

Chapter 2

Construction of Photo-Cross-Linked Microarrays of Small Molecules

Naoki Kanoh

Abstract

Small molecule microarrays are one of the most promising approaches to screen ligand molecules for individual proteins of interest. However, their potential has not been fully realized due to the limited number of methods to introduce small molecules onto the solid surfaces. To expand the compatibility of small molecule microarrays, we have developed a unique photo-cross-linking approach for immobilizing various small molecules, including natural products, on glass slides.

Key words: Photo-cross-linking, Natural products, Diazirine, Photo-generated carbene

Abbreviations

DSC	*N,N′*-disuccinimidyl carbonate
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
rt	Room temperature
UV	Ultraviolet light

1. Introduction

Construction of small molecule microarrays has mainly been accomplished using the selective coupling approach. That is, a library of small molecules having a certain functional group (e.g., thiol) is printed onto solid surfaces derivatized with a different functional group (such as maleimide) that reacts selectively with the former one. Thus, the library molecules readily attach to the surface through a coupling reaction (1). However, to construct small molecule microarrays by using this protocol, one must synthesize a library of compounds having a certain functional group,

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_2,

or select such compounds from an in-house library. Natural product libraries, which usually consist of a range of different types of molecules, are therefore considered to have poor compatibility with this platform. However, a method having high compatibility with functional groups would avoid this problem.

There is another drawback in using the selective coupling approach. Introduction of a tether, which connects a small molecule with a solid surface, on a defined site of a small molecule reduces the number of binding modes available for each compound. A decrease in the number of binding modes poses a major drawback to ligand screening and chemical genomics, especially when the goal is to discover new interactions between small molecules and proteins of interest.

To overcome these drawbacks of the selective coupling approach, we developed a unique "non-selective" photo-cross-linking protocol for immobilizing a variety of small molecules, including natural products, on glass slides (2, 3).

2. Materials

2.1. Preparation of Photo-Cross-Linker-Coated Glass Slides

1. Amine-coated glass slide: HA-coated slide glass for DNA microarray (Matsunami Glass Industries, Ltd., Osaka, Japan).
2. Gap cover glass (Matsunami Glass Industries, Ltd., Osaka, Japan).
3. Slide staining chamber (BT-220; Matsunami Glass Ind., Ltd., Osaka, Japan).
4. MildMixer XR-36 instrument (Taitec Co., Ltd., Saitama, Japan).
5. Spin Dryer Mini (Wakenyaku Co., Ltd., Kyoto, Japan).
6. Slide activation solution: Prepare a solution of *N,N′*-disuccinimidylcarbonate (100 mM) and *N,N*-diisopropylethylamine (100 mM) in reagent-grade *N,N*-dimethylformamide (DMF) prior to use.
7. Reagent grade ethanol.
8. Milli-Q water.
9. Photo-cross-linker solution: Prepare a solution of photo-cross-linker (compound 1 in Fig. 1, see Note 1) (100 mM) and *N,N*-diisopropylethylamine (500 mM) in reagent-grade DMF.
10. Blocking solution: 2-ethanolamine (1 M) in reagent-grade DMF.

2.2. Small Molecule Printing and Immobilization

1. Library of small molecules.
2. 384-well polypropylene plate (X6004; Genetix LTD., New Milton, UK).

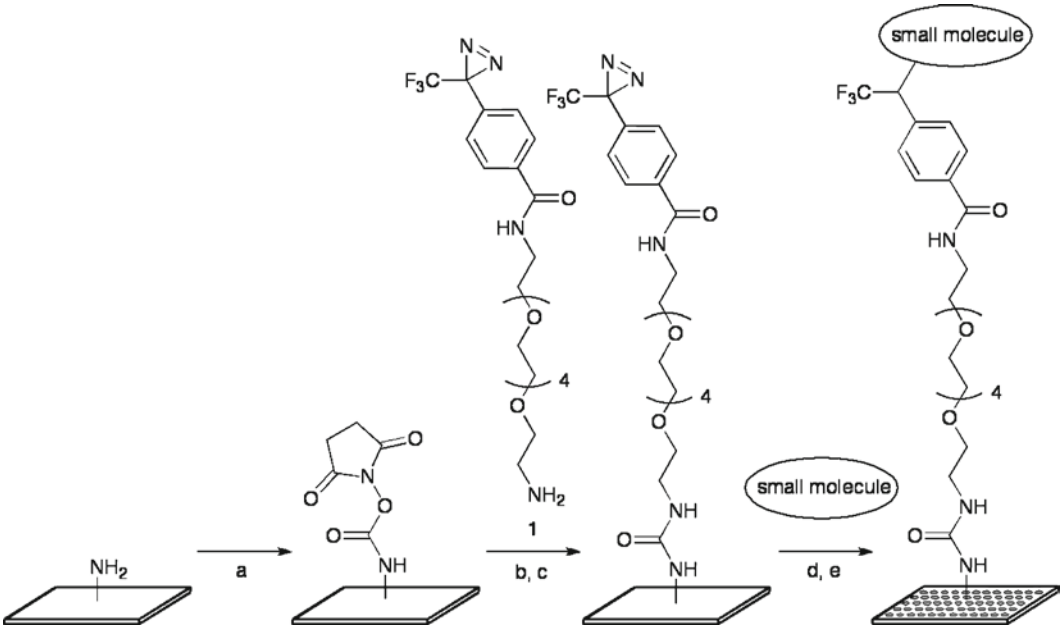


Fig. 1. Construction of photo-cross-linked microarrays of small molecules. Reagents and conditions: (**a**) DSC (100 mM), DIPEA (100 mM), DMF, rt, 4 h; (**b**) photo-cross-linker **1** (100 mM), DIEPA (500 mM), DMF, rt, dark, 4 h; (**c**) ethanolamine (1 M), DMF, rt, dark, 1 h; (**d**) print the small molecules, then dry; (**e**) UV (365 nm, 4 J/m^2).

3. TJ-25 Centrifuge (Beckman Coulter, Inc., CA).
4. Microplate holders (S5700; Beckman Coulter, Inc., CA).
5. CL-1000L ultraviolet cross-linker (UVP LLC, Upland, CA).
6. Reagent-grade DMSO.
7. Reagent-grade DMF.
8. Reagent-grade THF.

3. Methods

The present photo-cross-linking strategy for immobilizing small molecules depends on the reactivity of carbene species generated from a 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoic acid derivative upon UV irradiation. The photo-generated carbene species are known to be highly reactive toward a variety of chemical bonds, including nonactivated C-H bonds (4, 5). This property is important for immobilizing small molecule through multiple sites on the molecule to generate multiple conjugates. Generation of multiple conjugates from a small molecule is expected to retain the number of binding modes available for each molecule.

However, we (6) and others (7) have shown that, when the UV irradiation is done in solution phase, the photo-generated carbene species tend to react preferentially with a certain functional group over others. In addition, solvent molecules themselves can react with the carbene species, lowering the density of small molecules photo-cross-linked on the solid surface. Therefore, to maximize the cross-linking efficiency and randomness toward functional groups, we carry out the immobilization in a semisolid and highly concentrated state: That is, each small molecule solution spotted as an array is allowed to dry and concentrate to form a thin layer of the compound on the solid surface prior to photolysis.

3.1. Preparation of Photo-Cross-Linker-Coated Glass Slides

1. Immerse amine-coated glass slides (~10 slides) in a slide staining chamber, which is filled with slide activation solution.
2. Shake the chamber gently on a MildMixer XR-36 instrument or common bench top shaker at room temperature (rt) for 4 h.
3. Pour off the slide activation solution carefully from the chamber. Wash the activated slides briefly by pouring reagent-grade EtOH into the chamber and discarding the EtOH shortly after.
4. Wash the slides in the chamber successively with EtOH, Milli-Q, EtOH, and Milli-Q for 10 min each, and then shake one by one in Milli-Q in a beaker. After washing, centrifuge the slides (480 × *g*, 1 min) using a Spin Dryer Mini or common 2-slide centrifuge for microarray substrate.
5. Clean the surface of the activated glass slides by gently blowing away dust with an air sprayer.
6. Place the activated glass slides on a clean flat surface such as a lab bench top with the compound-to-be-spotted side facing upward. Then, carefully place a gap cover glass at the center of each activated glass slide.
7. Pipette 40–50 μL of photo-cross-linker solution and diffuse it slowly between the cover glass and activated glass slide.
8. Incubate the glass slides for 4 h in the dark.
9. Immerse the slides carefully in an EtOH-filled slide staining chamber. Tilt the slide glass in EtOH in the chamber to allow the cover glass to fall off (see Notes 2 and 3).
10. Immerse the slides successively in EtOH and water. Centrifuge them at 480 × *g* for 1 min.
11. Immerse the slides in a slide staining chamber filled with blocking solution.
12. Gently shake the chamber on a MildMixer XR-36 instrument or the like at rt for 1 h.
13. Wash the blocked slides successively with EtOH and Milli-Q. Dry the slides at 480 × *g* for 1 min using a Spin Dryer Mini or

the like. Package the dried slides in five-slide boxes, then place the boxes in vacuum sealer bags and seal them using a vacuum sealer. Store the slides at −20°C.

3.2. Small Molecule Printing and Immobilization

1. Dissolve small molecules in DMSO, typically 10 mM, or a range of 5–10 mg/mL.
2. Transfer 10 μL of each stock solution to individual wells in a 384-well polypropylene plate. A liquid transfer robot can be used if there are a large number of samples.
3. Centrifuge compound plates at 400 × *g* for 30 min using a TJ-25 Centrifuge or standard bench top centrifuge with microplate holders.
4. Place the activated slides onto the microarrayer platform.
5. Print compounds in the desired array format.
6. After the print run is completed, leave the printed slides overnight in the dark.
7. Place the slides in a CL-1000L ultraviolet cross-linker. Irradiate the slides at 365 nm with 4 J/cm^2.
8. Place the irradiated slides in a slide staining chamber. Immerse them in EtOH and wash for 1 h with shaking. Repeat the wash with DMF, THF, EtOH, and Milli-Q water (see Note 4).
9. Centrifuge the washed slides at 400 × *g* for 4 min.

3.3. Notes

1. The photo-cross-linker can be prepared in two steps from 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoic acid and *N*-(*tert*-butoxycarbonyl)-3,6,9,12,15-pentaoxaheptadecane-1,17-diamine by using the previously reported procedures (2). 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoic acid can be purchased from Bachem AG (Bubendorf, Switzerland). *N*-(*tert*-butyloxycarbonyl)-3,6,9,12,15-pentaoxaheptadecane-1,17-diamine cannot currently be obtained from commercial suppliers, but can be synthesized in four steps from hexaethylene glycol (EG6) by using the protocol reported in the literature (8). The synthesis should be carried out by trained chemists in facilities for chemical synthesis.
2. Moving the cover glass on the slide glass or removing it from the slide glass outside the liquid will harm the slide glass surface.
3. The photo-cross-linker can be recovered by concentrating the EtOH wash and purifying the residue via amine-coated silica gel.
4. It should be noted that, upon concentration of the small molecule solution and photolysis on the solid surface, some compounds stick to the solid surface and can be very difficult to remove using the usual washing protocol. To enhance the

slide washing process, sonication can be employed. In such cases, the slides are sonicated with a US Cleaner (As One Co., Osaka, Japan) or the like successively in DMSO, DMF, acetonitrile, tetrahedrofuran, dichloromethane, EtOH, and Milli-Q water (3 × 5 min each).

Acknowledgments

The author would like to thank Ms. Kaori Honda for technical assistance and critical comments. This work was supported by Grant-in-Aid for Young Scientists (A) No. 19681023 from the MEXT, Japan.

References

1. MacBeath, G., Koehler, A.N. & Schreiber, S.L. (1999) Printing small molecules as microarrays and detecting protein-ligand interactions en masse. J. Am. Chem. Soc. 121, 7967–7968.
2. Kanoh, N., Kumashiro, S., Simizu, S., Kondoh, Y., Hatakeyama, S., Tashiro, H. et al. (2003) Immobilization of natural products on glass slides by using a photoaffinity reaction and the detection of protein-small-molecule interactions. Angew. Chem. Int. Ed. 42, 5584–5587.
3. Kanoh, N., Asami, A., Kawatani, M., Honda, K., Kumashiro, S., Takayama, H. et al. (2006) Photo-cross-linked small-molecule microarrays as chemical genomic tools for dissecting protein- ligand interactions. Chem. Asian J. 1, 789–797.
4. Platz, M., Admasu, A.S., Kwiatkowski, S., Crocker, P.J., Imai, N. & Watt, D.S. (1991) Photolysis of 3-aryl-(trifluoromethyl) diazirines: A caveat regarding their use in photoaffinity probes. Bioconjugate Chem. 2, 337–341.
5. Nassal, M. (1984) 4′-(1-Azi-2,2,2-trifluoroethy)phenylalanine, a photolable carbene-generating analogue of phenylalanine. J. Am. Chem. Soc. 106, 7540–7545.
6. Kanoh, N., Nakamura, T., Honda, K., Yamakoshi, H., Iwabuchi, Y. & Osada, H. (2008) Distribution of photo-cross-linked products from 3-aryl-3-trifluoromethyldiazirines and alcohols. Tetrahedron 64, 5692–5698.
7. Tomioka, H. (1994) Reaction of carbenes in solidified organic molecules at low temperature. Res. Chem. Intermed. 20, 605–634.
8. Larsson, A., Angbrant, J., Ekeroth, J., Månsson, P. & Liedberg, B. (2006) A novel biochip technology for detection of explosives-TNT: Synthesis, characterisation, and application. Sens. Actuator B-Chem. 113, 730–748.

Chapter 3

Small Molecule Microarray: Functional-Group Specific Immobilization of Small Molecules

Hyang Yeon Lee and Seung Bum Park

Abstract

Proteomic screening with small molecule microarrays can be a powerful tool in conjunction with various forward chemical genetics screening and high-throughput phenotype assays. Small molecule microarray screening can provide high quality information from the direct binding interaction between proteins of interest and a collection of small molecules in a high-throughput fashion. To realize this potential of small molecule microarray in the postgenomic era, the immobilization of small molecules on the surface of microscope glass slides has been a critical step, to apply small molecule library in protein screening assays and dissecting the protein network. In this chapter, we would like to focus on the protocol for the systematic immobilization of synthetic drug-like small molecules containing either specific functional handles or common functional groups.

Key words: Small molecule microarray, Proteomic screening, Specific immobilization, Functional group specificity, Surface chemistry

1. Introduction

After the completion of Human Genome Project in 2003, Genetics/Genomics and Proteomics approach are in the limelight. There are a handful of new technologies, such as PCR, siRNA, recombinant protein expression, mass spectrometry, and so forth, which are revolutionizing research in biological and medical science. The perturbation of biological system is one of the key approaches to understand the function of particular gene products or biopolymers, and the perturbation on the biological systems can be poor nutrients, temperature variation, random mutation, and introduction of new gene products through plasmid/siRNA. One of the methods to perturb biological systems is the treatment of bioactive small molecule, which has been used

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_3,

for thousand years by human being as a medicine. Chemical genetics aims to understand the properties of nucleic acids and proteins using bioactive small molecule in a systematic fashion. The phenotype changes of certain organisms or the functional modulation of particular proteins of interest can be induced upon the treatment with bioactive small molecules, which provide powerful clues to understand the functions of particular proteins and their relationship with phenotypic changes in certain organisms.

Proteomic screening with small molecule microarrays can be a powerful tool in conjunction with various forward chemical genetics screening and high-throughput phenotype screening. Particularly, small molecule microarray screening can provide high quality information from the direct binding interaction between proteins of interest and a collection of small molecules in high-throughput fashion. Akin to DNA microarrays, small molecule microarrays could serve as an essential research tool for the understanding of the mode of action in various bioactive small molecules. To realize this potential in a research setting, the immobilization technology of small molecules on the surface of microscope glass slides has been a critical step for the application of small molecule microarrays. There is a huge interest on the application of small molecule microarray using natural products, natural-product analogs, and synthetic small molecule libraries from various sources. Unlike DNA, small molecules do not share common functional groups as a handle for immobilization onto the glass surface. In particular, natural products and natural-product analogs are well known for their structural and functional-group diversity around their core skeletons. Therefore, they require a universal functionalization method without any functional handles, which presents certain advantages and disadvantages. In this chapter, we would like to focus on the systematic immobilization of synthetic drug-like small molecules with shared functional groups.

In order to apply small molecule microarray in proteomic screening, chemical chips by the immobilization of hundreds of small molecules on microscope glass slides can provide the proper display of small molecules on the glass surface for the interaction with proteins of interest. There are several chemical methods for the functional group-based specific immobilization on the glass surface: Thiol-specific immobilization on maleimide-derivatized slide via Michael addition (1), primary alcohol-specific immobilization on silyl chloride-derivatized slide (2), diazobenzylidene-mediated immobilization of functional groups with acidic protons, such as phenols and carboxylic acids (3) (Fig. 1).

Even though these methods can provide a versatile tool for the selective immobilization of drug-like small molecules, they take advantage of a biased orientation of small molecule on the

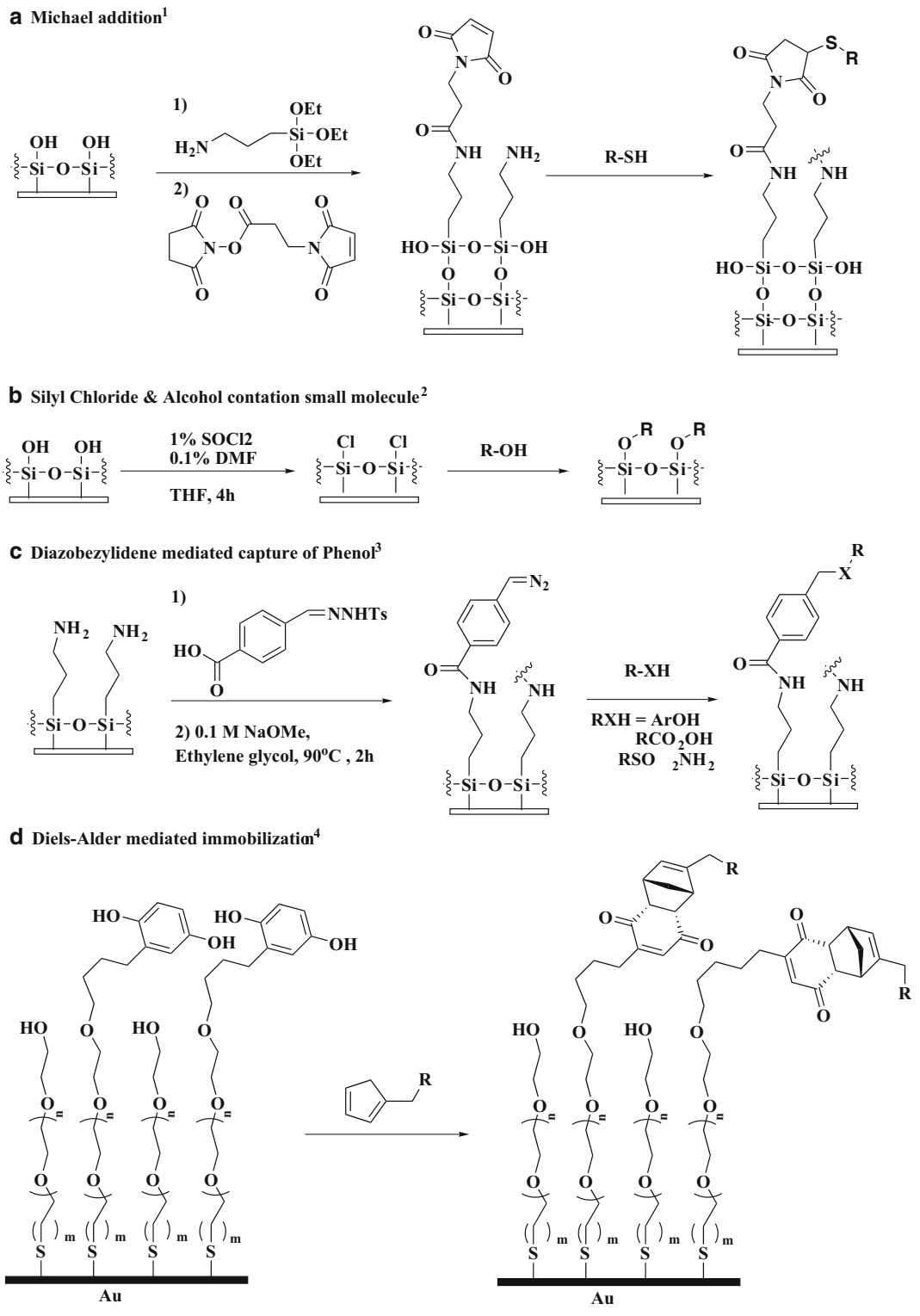
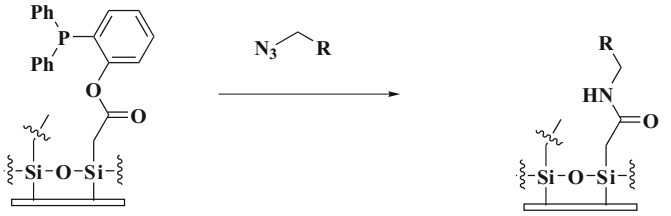
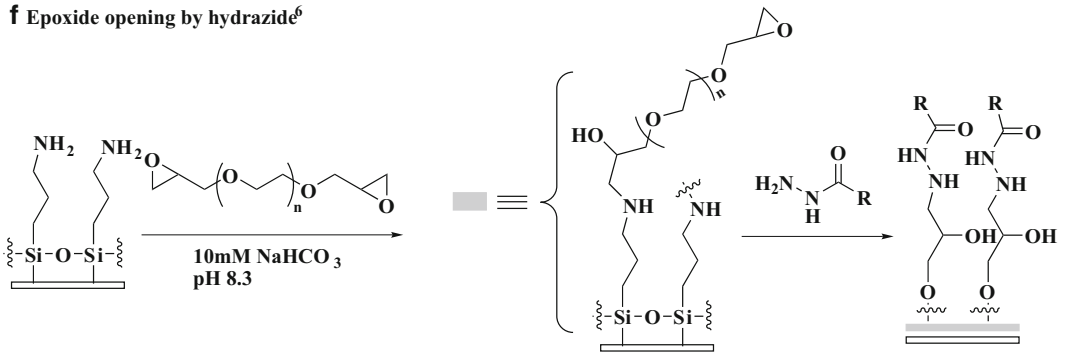


Fig. 1. Specific immobilization methods of small molecules containing various functional groups.

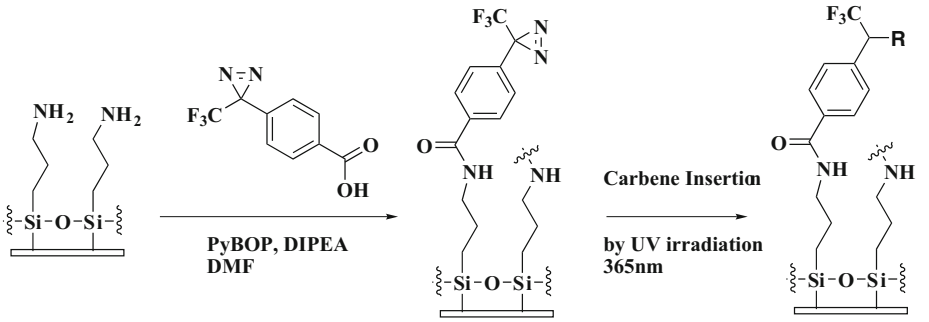
e Staudinger reaction[5]



f Epoxide opening by hydrazide[6]



g Photo affinity- Carbene insertion[9]



h Isocyanate-Pyridine vapor activation[10,11]

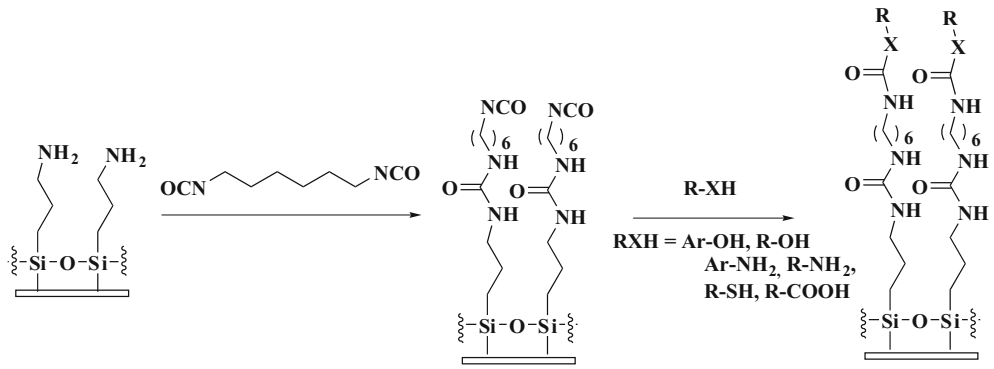


Fig. 1. (continued)

glass surface because of their selective reactivity toward certain functional groups. These small molecules require the common functional handles for the application in particular surface immobilization, for instance, a thiol group on the libraries is required for immobilization onto maleimide slides, primary alcohol for silyl chloride slides, and phenolic alcohol and carboxylic acids for diazobenzylidene slide. Sometimes, the introduction of specific functional groups on various small molecules can be achievable through combinatorial approach and diversity-oriented synthesis. In particular, the solid phase parallel synthesis can lead to the production of small molecule collection with the appropriate functional handle. But in the case of complex molecules, such as natural products, it is difficult to have a common functional handle or introduce such a functional group. To accommodate those small molecule collections with various functional groups, isocyanate-derivatized glass slide with pyridine vapor activation was developed (10, 11). Isocyanate slide and pyridine vapor activation provide a useful immobilization method which could be applied to small molecules with various nucleophilic functional handles. In addition, there is a list of other functional group-selective immobilization methods via specific reactions as following, which is not covered in this chapter: Diels-Alder reaction (4), Staudinger reaction (5), epoxide opening by hydrazide (6, 7), Biotin-Avidin capturing (8), and photo-activated crosslinking using diazirin (9). Most of these methods, except photo-activated crosslinking, take advantage of a biased orientation of small molecule on the glass surface (Fig. 1).

As stated earlier, the isocyanate method can be utilized for the surface immobilization of natural products or natural product-like small molecule library because this method can accommodate a wide spectrum of nucleophilic functional groups on small molecules (10, 11). Using this method, the nucleophilic functional groups, such as thiols, 1°/2° amines, 1°/2°/3° alcohols, anilines, phenols, sulfonamide, and even carboxylic acid, can be selectively immobilized on the isocyanate-derivatized glass surface. The binding event between small molecules and proteins of interest can be monitored with enhanced signal-to-noise ratio by the surface modification with short polyethylene glycol linkers through the reduction of nonspecific binding. On the other hand, the photo-activated crosslinking method was developed for the universal immobilization of small molecules on the glass surface using nonselective carbene insertion chemistry. Using this method, small molecule with any functional groups can be immobilized through random insertion of reactive species, such as carbene, nitrene, or radical, freshly generated on the glass surface after small molecule printing. This immobilization chemistry is a very powerful tool to introduce any type of small molecule, even simple hydrocarbons.

However, this photo-activated crosslinking method is also challenged by the issues on false positives caused by the untraceable structural perturbation during the random insertion chemistry. Thus, the development of mild and compatible fabrication methods is important to apply natural product and natural product-like small molecule library to small molecule microarray. This immobilization chemistry is covered herein. In this book chapter, we focus on the protocol for the systematic immobilization of synthetic drug-like small molecules containing either specific functional handles or common functional groups and suggest the proper immobilization method for specific functional group.

2. Materials

2.1. Preparation of Small Molecule Source Plates

1. Small molecule with various functional handles (for proof-of-concept experiment).
2. Dimethyl sulfoxide (DMSO).
3. 384-well microtiter plate (polypropylene, GenePix or equivalent, molecular devices, USA).
4. Plate sealing tape (Thermowell™ Sealing Tape, Corning, USA).
5. Sealed container (i.e., Lock & Lock) with blue silica gel.

2.2. Preparation of Amine-Functionalized Glass Slides

1. Prepared glass slides (1″×3″, 1 mm thickness, Corning, USA).
2. Piranha solution: a mixture of concentrated sulfuric acid and 30% hydrogen peroxide solution (Aldrich, USA) in 70:30 (v/v) (see Note 2).
3. (3-aminopropyl)triethoxysilane (Aldrich, USA).
4. 95% ethanol.
5. Dry oven for curing slides.

2.3. Preparation of Chlorinated Glass Slide

1. Prepared glass slides (1″×3″, 1 mm thickness, Corning, USA).
2. Piranha solution: a mixture of concentrated sulfuric acid and 30% hydrogen peroxide solution (Aldrich, USA) in 70:30 (v/v) (see Note 2).
3. Thionyl chloride ($SOCl_2$, Aldrich, USA).
4. *N,N*-Dimethyformamide(DMF).
5. Tetrahydrofuran (THF).

2.4. Preparation of Surface Modification on Amine-Functionalized Glass Slides

1. *N*-succinimidyl 3-maleimido propionate (Aldrich, USA).
2. Dimethylformamide (DMF).
3. 50 mM sodium bicarbonate buffer, pH8.5.
4. 2-mercaptoethanol (Aldrich, USA).
5. Tetrahydrofuran.
6. Iisopropanol.

2.4.1. Maleimide-Functionalized Glass Slides

2.4.2. Diazobenzylidene-Derivatized Glass Slides

1. Toluenesulfonylhydrazone [synthesized from 4-carboxybenzaldehyde (Alfa Aesar, USA)].
2. p-Toluenesulfonylhydrazide (Aldrich, USA).
3. Methanol.
4. (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP, Novabiochem).
5. *N,N*-diisopropylethylamine (DIPEA).
6. Sodium methoxide (Aldrich, USA).
7. Ethylene glycol (Junsei, Japan).
8. Dimethylformamide.

2.4.3. Isocyanate-Derivatized Glass Slide

1. 1,6-diisocyanatohexane (Acros, USA).
2. Dimethylformamide (DMF).
3. Fmoc-8-amino-3,6-dioxaoctanoic acid [synthesized in-house using reported procedure or purchased from Fluka, USA].
4. (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP, Novabiochem, Germany).
5. *N,N*-diisopropylethylamine (DIPEA).
6. 20% (v/v) Piperidine in DMF.

2.5. Printing Small Molecule Microarrays

1. OmniGrid Microarrayer (Digilab Inc., USA) or equivalent microarrayer.
2. Stealth microspotting pins (SMP-5 pin, ArrayIt Corp., USA).
3. Pin washing with acetonitrile and sonication with DMF.
4. Vacuum oven (Eyela, Japan) and pyridine (Aldrich, USA) for isocyanate chemistry activation.
5. 1 M ethylene glycol (Junsei, Japan) or glycolic acid (Aldrich, USA) in DMF for quenching the residual microarray surface.

2.6. SMM Incubation with Proteins of Interest

1. Phosphate-buffered saline with Tween-20 (PBST) : 12 mM NaH_2PO_4, 137 mM NaCl, 3 mM KCl, pH = 7.4, 0.01 % (v/v) Tween-20.
2. Proteins of interest in appropriate storage buffer.

3. Parafilm or Saran wrap (clear plastic wrap).
4. Cy3/Cy5 monoreactive protein labeling dye (GE Healthcare, UK) for fluorescent labeling on protein of interests or antibody.
5. Streptavidin (Aldrich, USA) as an example of proteins of interest.
6. FKBP12-GST (in-house expressed using recombinant protein purification technology).
7. Anti-GST monoclonal antibody (GE Healthcare, UK) labeled with Cy5 fluorophores.

2.7. Fluorescent Scanning

1. Axon GenePix 4200B 2-laser microscope slide scanner (Molecular Devices, USA) or equivalent fluorescent microscope slide scanner.
2. GenePix Pro 5.0 software (Molecular Devices, USA).

3. Methods

For the practical application of small molecule microarray in proteomic screening, it is required to fabricate chemical chips by the immobilization of thousands of small molecules on glass slides, which can provide the proper display of small molecules on the glass surface for the interaction with proteins of interests. There are several chemical methods for the functional group-based specific immobilization on the glass surface. In this chapter, we focus four immobilization methods: Thiol-specific immobilization on maleimide-derivatized slide via Michael addition (1), primary alcohol-specific immobilization on silyl chloride-derivatized slide (2), diazobenzylidene-mediated immobilization of functional groups with acidic protons, such as phenols and carboxylic acids (3), and semiuniversal immobilization of various nucleophilic functional groups using isocyanate-derivatized slide with pyridine vapor activation (10, 11). First of all, thiol-containing small molecules can be immobilized on the maleimide-derivatized glass slides via Michael addition reaction of the strong nucleophile (thiol) to the electrophile (α,β-unsaturated carbonyl) (1). In comparison, primary alcohol-containing small molecules can selectively immobilized on the silyl chloride-derivatized glass slides, due to the oxophilicity of silicon moiety. Silyl chloride group can be introduced by the treatment of thionyl chloride on the freshly reduced silyl alcohol (with Piranha solution) (2). However, small molecules with phenolic alcohol cannot be immobilized effectively on the silyl chloride glass surface. To address this problem, a new immobilization

method was developed: small molecules containing functional groups with acidic protons, such as phenolic alcohol and carboxylic acid, can be immobilized on the diazobenzylidene-derivatized glass slides (3). This method can provide the complementary tool to immobilize phenolic alcohol-containing small molecules which are not applicable in silyl chloride glass slides. To accommodate small molecule collections with various functional groups, isocyanate-derivatized glass slide with pyridine vapor activation was developed (10, 11). Isocyanate slide and pyridine vapor activation provide a useful immobilization method which could be applied to small molecules with various nucleophilic functional handles, such as amine/aniline, 1°/2°/3° alcohol, phenol, and thiol, etc. In general, isocyanate can react with many nucleophilic functional groups under the base-catalyzed condition. The isocyanate chemistry was successfully introduced on the surface immobilization method for a series of small molecules with various functional handles through the pyridine vapor activation in the vacuum oven for the surface. Herein, we introduce these small molecule fabrication methods according to the functional group using FKBP12 as a target protein and AP1497 derivatives as a small molecule. (see Note 1)

3.1. Preparation of Small Molecule Source Plates

1. Dissolve small molecules in DMSO at the concentration of 10 mM. For the proof-of-concept experiment and sensitivity profiling, the control molecules (biotin or AP1497) with various functional handles in DMSO solution were serially diluted from 10 to 0.625 mM.
2. Transfer 10 μL of each solution to 384-well polypropylene plate as small molecule source plate. Seal each plate with plate sealing tapes and store them at –20°C until use. The small molecule source plates are recommended to be stored in the sealed container with blue silica gel to minimize the hydration of DMSO solution during freeze and thaw cycles.

3.2. Preparation of Amine-Functionalized Glass Slides

1. Prepare piranha solution as following: a mixture of concentrated sulfuric acid (Aldrich, USA) and 30% hydrogen peroxide solution (Aldrich, USA) in 70:30 (v/v) (see Note 2).
2. Immerse the plain glass slides into the piranha solution for 12 h at room temperature under the fume hood for the generation of fresh silanol on the glass surface.
3. Wash the resulting glass slides briefly with dH_2O and immerse them into the solution of (3-aminopropyl)triethoxysilane (3% v/v) in 95% ethanol for 1 h at room temperature.
4. Gently swirl the resulting glass slides in absolute ethanol to remove excess (3-aminopropyl)triethoxysilane and incubate them in the dry oven at 115°C for 1 h to cure the absorbed silane layer.

5. Wash amine-functionalized glass slide extensively with DMF, acetonitrile and dH_2O. Dry the resulting slides by centrifugation (23× *g*) and store them under nitrogen or argon gas.

3.3. Preparation of Maleimide-Derivatized Glass Slides (see Figs. 2 and 3)

1. Prepare amine-functionalized glass slide or commercial GAPS (γ-aminopropylsilane) slide.
2. Prepare maleimide solution for the functionalization of amine-functionalized slide as following: Dissolve *N*-succinimidyl 3-maleimido propionate in anhydrous DMF (200 mM), and dilute this solution by tenfold with 50 mM sodium bicarbonate aqueous buffer (pH 8.5).
3. Immerse the amine-functionalized slides into the maleimide solution for 3 h at room temperature.
4. Wash the resulting slides extensively with DMF, acetonitrile and dH_2O and dry them by centrifugation (23× *g*).

3.4. Small Molecule Printing

1. Place the prepared 384-well plate containing small molecule stock solution in DMSO at the source plate position in the microarrayer.

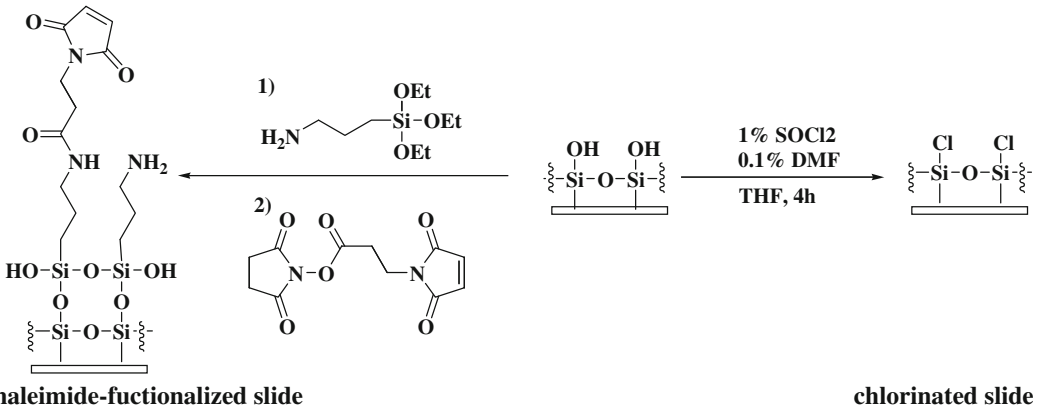


Fig. 2. Preparation of maleimide-functionalized and silyl chloride-derivatized glass slide.

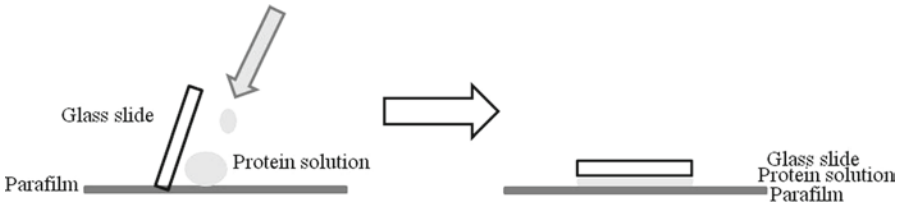


Fig. 3. Schematic diagram of SMM incubation with proteins of interest using parafilm. Protein solution (500 uL) was dropped onto the parafilm and the printed face of small molecule microarray can gently cover the protein solution. The protein solution was diffused throughout the slide surface by capillary effect. The SMM incubation can be performed on the benchtop at room temperature incubation or in the cold room for 4°C incubation.

2. Install the small molecule printing pins on the pin-head of the microarrayer (2×2, 4×4, 4×12, etc.) and design the printing method and configuration using OmniGrid software: blot 40 features before printing on actual slides.
3. Place the functionalized glass slide on the platform of the microarrayer.
4. Put a clean glass blot pad on the microarrayer platform.
5. Print the small molecule on the surface of functionalize glass surface using the protocol developed by the manufacturer of the microarrayer. (number of pin blotting on the blotting pad, pin washing methods and sonication time, and pin drying, etc.).
6. Some immobilization technology requires postprocessing steps after the small molecule printing.

3.5. Protein Binding Assay (Proteomic Screening) with Small Molecule Microarray (see Fig. 4)

1. Prepare proteins of interest labeled with fluorophores (such as Cy3 and Cy5). The binding event of small molecule with proteins of interest can be monitored either by direct labeling of proteins of interest with fluorophores or by visualizing the proteins of interest with epitope tag using fluorescently labeled anti epitope primary antibody or secondary antibody. In this protocol, GST-fusion proteins were used for proteomic screening and their binding events were monitored by Cy3-labeled anti-GST primary antibody.

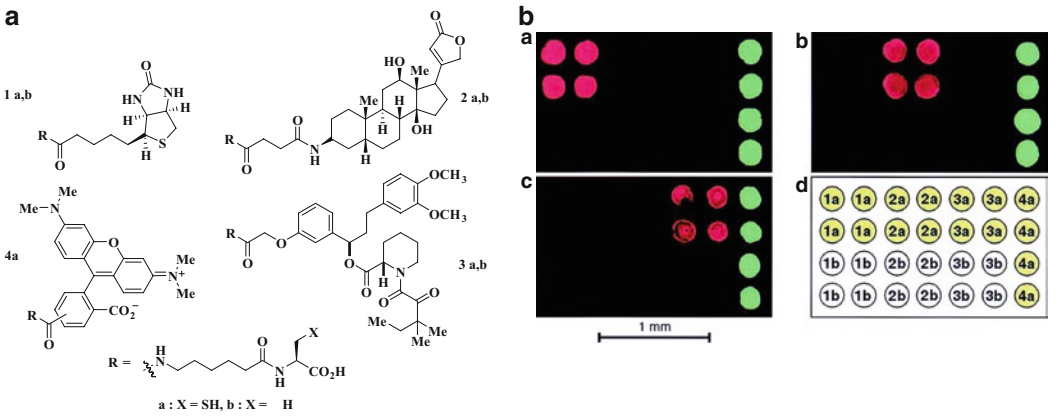


Fig. 4. Small molecules containing thiol group (**1a**, **2a**, **3a,** and **4a**) were selectively immobilized on maleimide-derivatized glass slides via Michael addition reaction. Compound **1a** and **1b** are biotin derivatives with two different functional handles. Biotin is one of the strongest small molecule binders toward avidin/streptavidin (estimated binding affinity K_D values of ~10^{-15} M). Compound **2a** and **2b** are steroid digoxigenin derivatives and are known as an antigen of mouse monoclonal antibody DI-22. Compound **3a** and **3b** are synthetic pipecolyl a-ketoamides (AP 1497) and can selectively recognize the human imminophilin FKBP12 with excellent affinity. Compound **4a** is a thiol-functionalized tetramethylrhodamine as a reference fluorophore. The binding events between small molecules and proteins were detected with fluorescent (Cy5) labeled proteins. (Reproduced from ref. (1) with permission from American Chemical Society).

2. Scan the slides before applying proteins of interest to determine any auto fluorescence of printed small molecule.
3. Immerse printed small molecule microarrays in PBST (see Note 3) containing 3% BSA and incubate them for 20 min at room temperature to block nonspecific interactions (see Note 4).
4. Immerse the slides into PBST containing 1% BSA with gentle shaking for 10 min at room temperature.
5. Wash slides with PBST and dry them by brief centrifugation (23× *g*, 30 s).
6. Prepare a solution with proteins of interest (typically 0.1 μg/mL ~5 μg/mL in PBST containing 1% BSA) (see Note 5).
7. Incubate the printed face of smallmolecule microarray with a protein solution (500 μL) for 30 min on the top of parafilm for 30 min at 4°C or room temperature (see Notes 6, 7 and 8) (see Fig. 3).
8. Wash the resulting slides with PBST containing 1% BSA (three times) for 20 min at room temperature. If it requires the second incubation with fluorescence-labeled primary antibody, incubate the slides with 500 μL of a solution of Cy5 labeled goat anti-GST antibody (1 μg/mL in PBST) for another 30 min.
9. Rinse them with PBST containing 1% BSA for 20 min at room temperature. Repeat twice.
10. After the final brief washing with dH_2O, dry the microarray slides by centrifugation (23× *g*, 30 s).
11. Scan the resulting slides with fluorescent microscope slide scanner (GenePix 4200A) (see Note 9).
12. Analyze the results of proteomic screening with the analysis software (GenePix Prof 5.0).

3.6. Preparation of Silyl Chloride-Derivatized Glass Slides (see Figs. 2 and 5)

1. Prepare piranha solution as following: a mixture of concentrated sulfuric acid and 30% hydrogen peroxide solution in 70:30 (v/v) (see Note 2).
2. Immerse the plain glass slides into the piranha solution for 12 h at room temperature under the fume hood for the generation of fresh silanol on the glass surface.
3. Wash the resulting glass slides briefly with dH_2O.
4. Immerse the slides into the chlorination solution which is composed of 1% $SOCl_2$ and 0.1% DMF in anhydrous THF for 4 h at room temperature.
5. Wash the slides briefly with anhydrous THF and dry them by centrifugation (23× *g*), and use the resulting slide immediately after the silyl chloride derivatization.

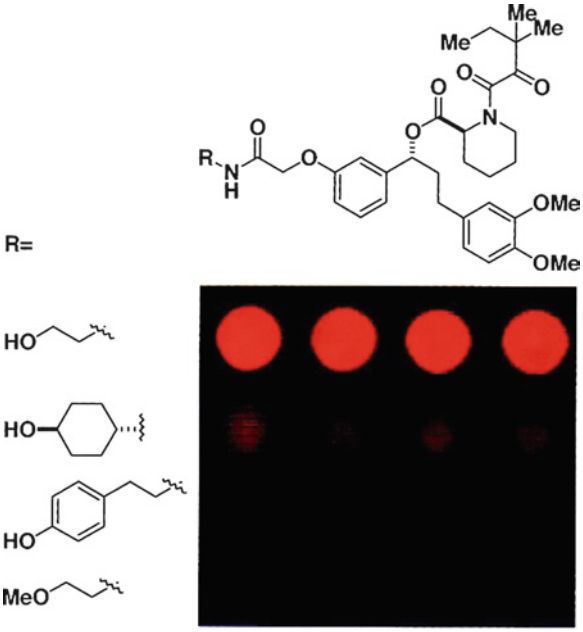


Fig. 5. AP1497 derivatives containing various functional handles, such as primary alcohol, secondary alcohol, phenolic, and methyl ether, were printed on silyl chloride-derivatized glass slides at the concentration of ~5 mM stock solution. The resulting slide was incubated with Cy5-labeled FKBP12 protein solution. AP1497 with primary alcohol was selectively immobilized on the silyl chloride-derivatized glass slide due to its reactivity with silyl chloride. In comparison, AP1497 with secondary alcohol showed weak fluorescent (Cy5) signal, which confirms the poor immobilization of secondary alcohol on the silyl chloride-derivatized glass surface. Phenolic and methyl ester functional handles failed to immobilize AP1497 on the surface of functionalized glass slide (Reproduced from ref. (2) with permission from American Chemical Society).

6. Print small molecules on the silyl chloride-derivatized glass surface, as described in Subheading 3.4, and leave the printed small molecule microarray at room temperature overnight for the proper immobilization. The optimal result can be achieved when the microarray printing is performed under the anhydrous N_2 atmosphere throughout the printing event.
7. Wash the resulting printed slides extensively with DMF, THF, acetonitrile, and water in sequence, and dry them by centrifugation (23× *g*, 30 s). The printed SMM can be stored at −20°C with argon purging for more than 6 month prior to the proteomic screening.
8. Perform the proteomic screening with proteins of interest, as described in Subheading 3.5 (see Fig. 5).

3.7. Preparation of Diazobenzylidene-Derivatized Glass Slides (see Figs. 6 and 7)

1. Synthesis of *p*-toluenesulfonylhydrazone: Dissolve 4-carboxybenzaldehyde (10 g, 66.7 mmol) and *p*-toluenesulfonylhydrazide (12.4 g, 66.7 mmol) in methanol (300 mL). The reaction mixture was heated at 70°C, then cooled down to 25°C and stirred for 16 h at room temperature. The resulting

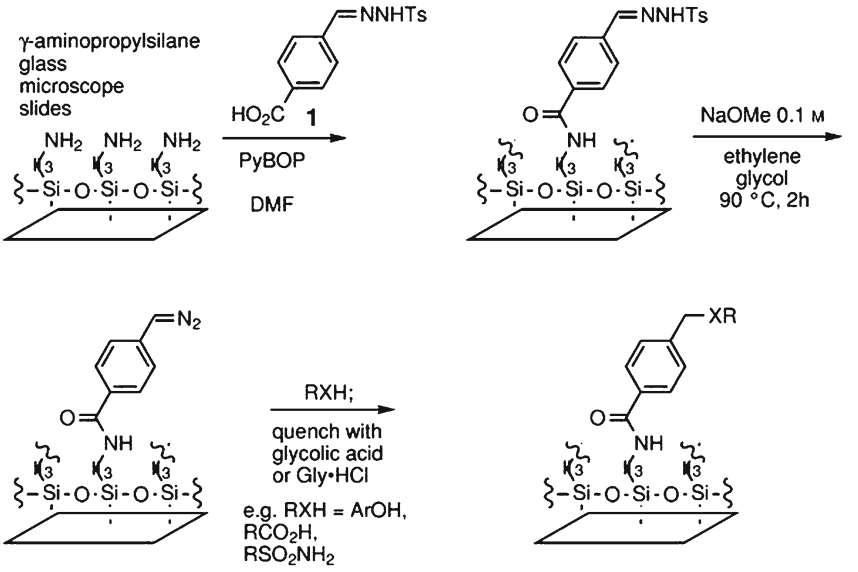


Fig. 6. Schematic diagram for the preparation of diazobenzylidene-derivatized glass slides followed by small molecule printing and quenching (Reproduced from ref. (3) with permission from Wiley-VCH Verlag GmbH & Co. KGaA).

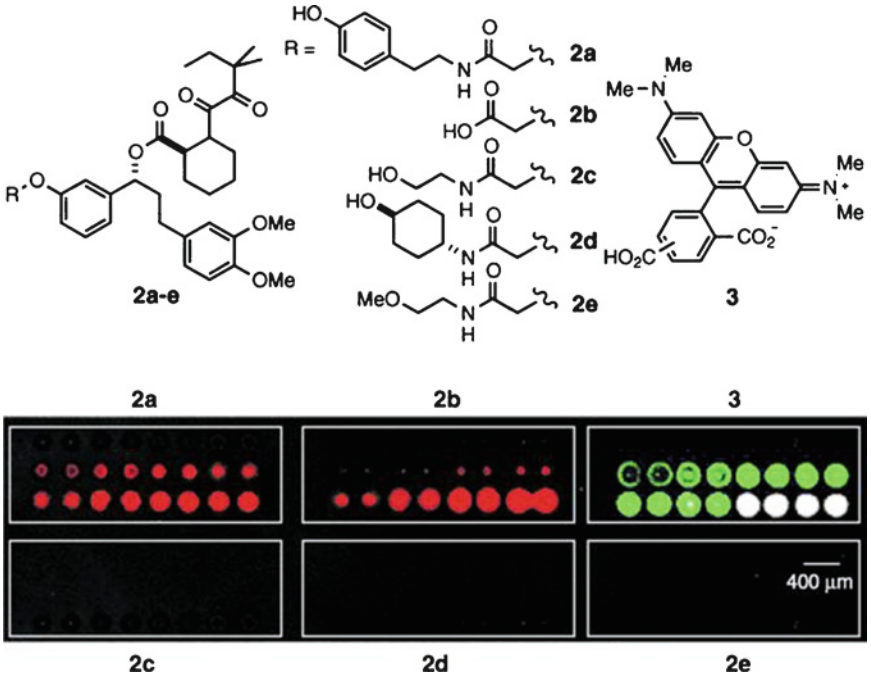


Fig. 7. AP1497 derivatized with functional handles, such as phenol (**2a**), carboxylic acid (**2b**), primary alcohol (**2c**), secondary alcohol (**2d**), methyl ether (**2e**), and tetramethylrhodamine (**3**) as a reference fluorophores were printed in a serial twofold dilution from 2 mM to 1 μM on diazobenzylidene-derivatized glass slides. The printed slide was incubated with Cy5-labeled FKBP12 (1 μg/mL) as proteomic screening. AP1497 derivatives containing phenolic OH and carboxylic acid were immobilized on the glass surface and successfully demonstrated the dose-dependent fluorescent intensity, which confirms that the immobilization efficiency of small molecules with proper functional handles is proportional to the concentration of small molecules. (Reproduced from ref. (3) with permission from Wiley-VCH Verlag GmbH & Co. KGaA).

mixture was heated again at 60°C, added with water (150 mL), and cooled down to 25°C. The desired product, *p*-toluenesulfonylhydrazone, as a white precipitate was filtered and washed with water. The additional water (400 mL) facilitates the formation of more precipitate from the filtrate. The white precipitate was collected and washed with cold water, and air-dried. *p*-Toluenesulfonylhydrazone was stored in the brown glass bottle at room temperature.

2. Immerse freshly prepared amine-functionalized slides or commercial GAPS slides into the solution of *p*-toluenesulfonylhydrazone (10 mM), PyBOP (10 mM), and DIPEA (20 mM) in DMF for 12 h at room temperature.
3. Wash the resulting slides extensively with DMF and methanol.
4. Immerse *p*-toluenesulfonylhydrazone-derivatized slides into the solution of 100 mM NaOMe in ethylene glycol, and incubate them at 90°C for 2 h for the generation of diazobenzylidene moiety on the surface of glass slides.
5. Wash slides with methanol and dry them by centrifugation (23× *g*, 30 s) (see Note 10).
6. Print small molecule on the surface of glass slides using microarrayer and leave the printed slide on the microarrayer platform for 12 h (overnight), as described in Subheading 3.4.
7. Immerse them in a 1 M aqueous solution of glycolic acid for 30 min to mask the remaining diazobenzylidene moieties.
8. Wash the resulting printed slides extensively with DMF, THF, methanol, and dH_2O in sequence and dry them by centrifugation (23× *g*, 30 s). The printed SMM can be stored at −20°C with argon purging for more than 6 month prior to the proteomic screening.
9. Perform the proteomic screening with proteins of interest, as described in Subheading 3.5 (see Fig. 7).

3.8. Preparation of Isocyanate-Coated Glass Slides (see Fig. 8)

1. Prepare the reaction solution containing Fmoc-8-amino-3,6-dioxaoctanoic acid (10 mM), PyBOP (10 mM), and DIPEA (20 mM) in 2.5 mL DMF for 5 amine-functionalized slides (GAPS II slide or equivalent slides).
2. Pipette the reaction solution (500 μL) onto the parafilm and place the GAPS II on the top of reaction solution, which allows the reaction solution to be spread throughout the surface of slides.
3. Incubate the slides with reaction solution at room temperature for 12 h.

4. Immerse the slides in fresh DMF and wash them for 10 min with gentle shaking. Repeat this with fresh DMF twice. The resulting slide is chemically modified with short polyethylene glycol (PEG) linker to minimize the nonspecific binding with biopolymers. After washing, slides can be dried by brief centrifugation (23× *g*, 30 s) and stored at room temperature prior to the following step.
5. Immerse the resulting slides in DMF solution containing 20% piperidine (v/v) for 30 min at room temperature to remove Fmoc protecting group on the surface of slides.
6. Immerse slides in fresh DMF and wash for 10 min with gentle shaking. Repeat with fresh DMF twice.
7. The resulting slides were dried by brief centrifugation and placed into a reaction jar (made of polypropylene).
8. Immerse slides in 10% (v/v) 1,6-diisocyanatohexane in DMF and incubate them for 30 min at room temperature with gentle shaking.
9. Wash the resulting slides in anhydrous DMF with gentle shaking for 5 min. Repeat this with fresh DMF and THF.
10. Dry slides by brief centrifugation (23× *g*, 30 s) and store at –20°C (see Note 11).

3.9. Printing of Small Molecules Using Microarrayer on Isocyanate-Functionalized Glass Slide and Immobilization Method Via Pyridine Vapor Activation (see Figs. 8 and 9)

1. Place the prepared 384-well plate containing small molecule stock solution in DMSO at the source plate position in the OmniGrid microarrayer.
2. Install the printing pins on the pin-head of the microarrayer (2×2, 4×4, 4×12, etc.), and design the printing method and configuration using OmniGrid software: prespot 40 features.
3. Place the isocyanate-functionalized glass slide on the platform of the microarrayer.
4. Put a clean glass blot pad on the microarrayer platform.

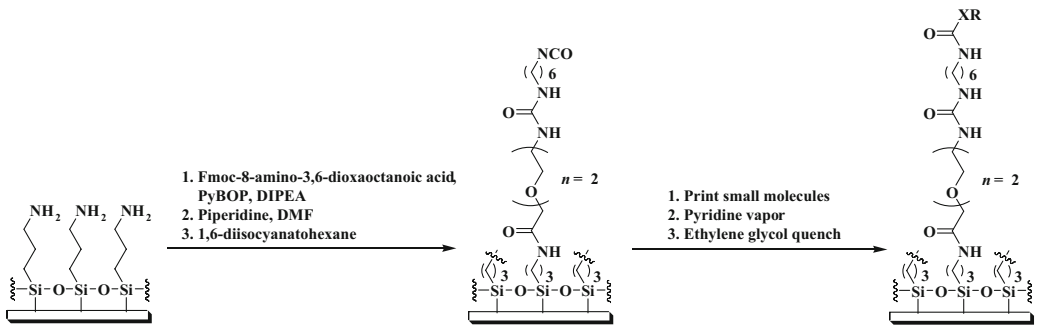


Fig. 8. Schematic diagram for small\-molecule immobilization on the isocyanate-derivatized glass slide and pyridine-vapor activation chemistry (Reproduced from ref. (10, 11) with permission from Nature Publishing Group).

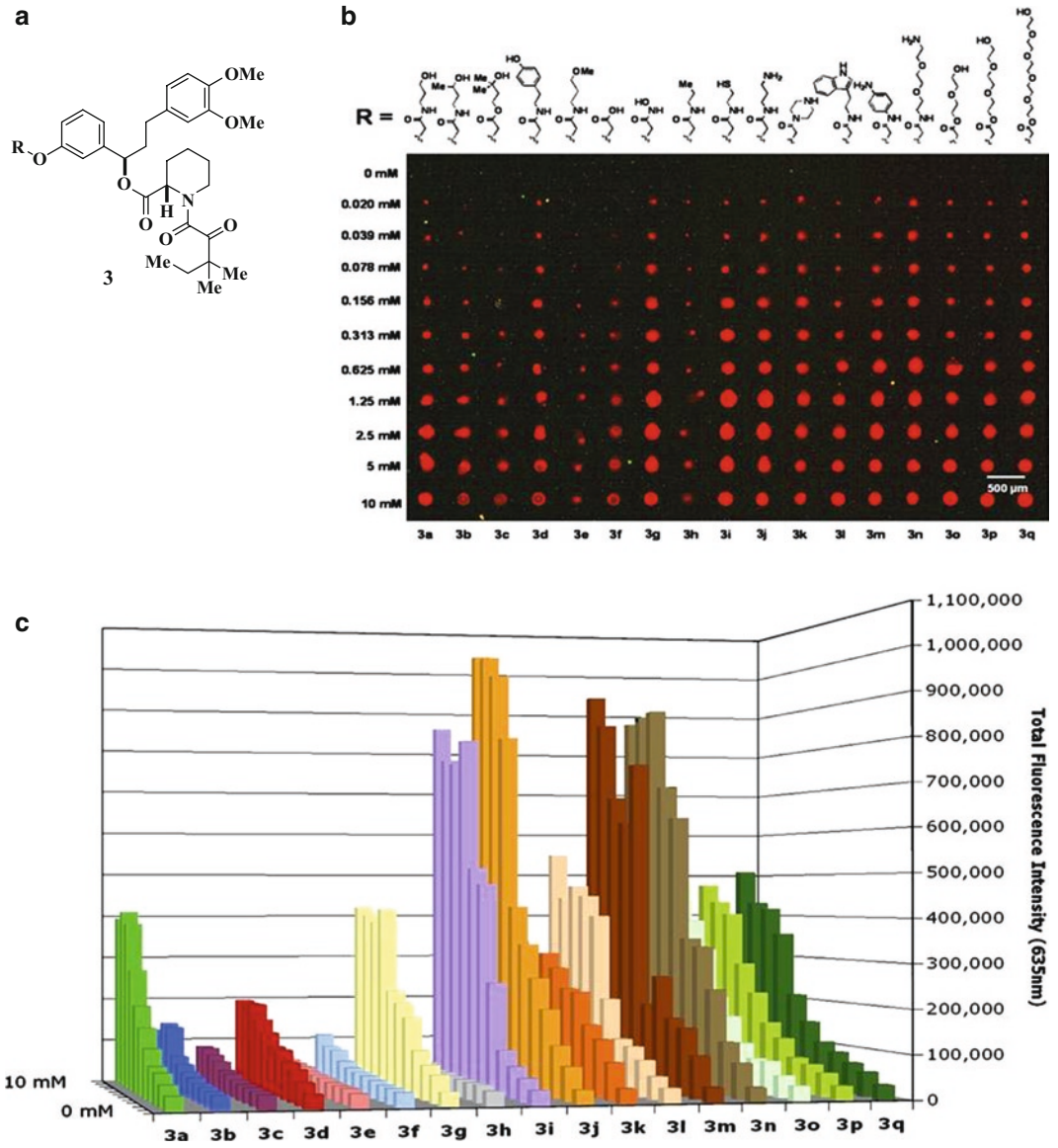


Fig. 9. AP1497 derivatives with various functional handles were printed on the isocyanate-functionalized glass slide followed by pyridine-vapor activation. Small molecule containing nucleophilic functional handles can successfully be reacted with electrophilic isocyanate and immobilized on the glass surface. Total fluorescence intensity was calculated by GenePix Pro analysis software. Primary amines (**3j**), aryl amine (**3m**), and thiol (**3i**) were immobilized with the highest immobilization efficiency. On the other hand, methyl ether (**3e**) and methyl (**3h**) were hardly immobilized. Small molecules containing various lengths of PEG linkers (**3o**–**3q**) did not make any significant difference in their fluorescent intensity based on their PEG chain length. (Reproduced from ref. (10, 11) with permission from Elsevier).

5. Print the small molecule on the surface of isocyanate-functionalized glass surface using the protocol developed by the manufacturer of the microarrayer (number of pin blotting on the blotting pad, pin washing methods and sonication time, and pin drying, etc.).

6. After the completion of print run, let the printed slides on the microarrayer platform for additional 10 min at room temperature.
7. Move the slides gently into the vacuum oven and close the door of the vacuum oven.
8. Heat the vacuum oven to 50°C and make the oven vacuous by opening the vacuum valves for 10 min.
9. Connect the two-neck round bottom flask (500 mL) containing 5 mL of Pyridine. One neck is connected to the vacuum oven, and the other neck is connected to the N_2 source (N_2 cylinder).
10. Fill the N_2 gas in the round bottom flask, and close both valves on the necks of round bottom flask.
11. Vaporize pyridine in the round bottom flask with heat-gun under isolated N_2 atmosphere. Then, open the valve to the vacuum oven and fill up the vacuous space of vacuum oven with pyridine vapor in N_2 gas.
12. Repeat step 11 until the inside of vacuum oven become atmospheric pressure. This operation allows the exposure of printed slides in the pyridine vapor with N_2 gas at 50°C. (see Note 12).
13. Close the valves (inlet and outlet) of vacuum oven and leave the printed slides under pyridine vapor activation at 50°C for overnight.
14. Immerse the printed slide into 1 M ethylene glycol in DMF solution to quench the residual isocyanate on the glass surface. Wash the resulting slides in DMF for 20 min with gentle shaking. Repeat the washing step for three times with fresh DMF.
15. Dry them by brief centrifugation (23× *g*, 30 s). The printed SMM can be stored at −20°C with argon purging for more than 6 month prior to the proteomic screening.
16. Perform the proteomic screening with proteins of interest, as described in Subheading 3.5 (see Fig. 9).

4. Notes

1. The affinity of AP1497 to the protein FKBP12 was reported around 8.8 nM.
2. Piranha solution is a very strong oxidant and highly corrosive. Thus, it should be handled with extreme care. Piranha solution is typically stored in glass containers and kept under the fume hood.

3. Proper buffer for the target protein should be used to maintain the activity and stability of the proteins of interest.
4. To prevent the nonspecific interaction with biopolymers, small molecule microarrays were incubated in 3% BSA / PBST (20 min) and 1% BSA/PBST (15 min × 2) solution prior to the incubation with target protein.
5. To reduce the nonspecific binding of protein, add 1% BSA (w/v) to the incubation buffer containing proteins of interest and test lower concentration of proteins.
6. The incubation temperature can be varied either at 4°C or at room temperature based on the stability and activity of the proteins of interest.
7. To reduce the amount of protein used for proteomic screening, parafilm method was used for protein binding assay. Transfer 500 μL of protein solution onto the parafilm and turn the slides upside down so that printed side can face the solution. Avoid the generation of air bubble (see Fig. 3).
8. When using the direct labeling, skip step 8. Fluorescent detection using common affinity tag antibody labeled with Cy3 or Cy5 can significantly reduce the labor and time to label individual proteins of interest. Thus, it can be applicable for the proteomic screening with cell lysates without further purification.
9. Choose the proper wavelength of fluorescent microscope slide scanner according to the fluorescence dye used for proteomic screening. The typical excitation wavelength of Cy3 and Cy5 are 532 nm and 635 nm, respectively.
10. Store the slides at −20°C prior to proteomic screening. However, they can be stored at this point for at least 3 weeks in the dark at room temperature without the loss of its activity.
11. The slides should be completely dried to maintain their reproducible binding events. So wash with DMF, THF, acetonitrile, and water, and then dry them by centrifugation (23× *g*, 30 s). After that, store them in −20°C until use. The activity was preserved for 6 months.
12. Pyridine vapor serves as a catalyst to increase the reactivity of various nucleophilic functional groups toward electrophilic isocyanate. To make pyridine anhydrous, potassium hydroxide was added into pyridine.

Acknowledgments

Our own investigations on proteomic screening and specific immobilization for small molecule microarray were supported by the National Research Foundation of Korea (NRF) and the WCU

program through NRF funded by the Korean Ministry of Education, Science and Technology (MEST). These colleagues whose names are mentioned in the references have contributed to our understanding of the surface modification and high-throughput screening using small molecule microarray. Last but not least, we are grateful to all group members of chemical biology laboratory at Seoul National University for their generous support and assistance.

References

1. MacBeath, G., Koehler, A. N., Schreiber, S. L. (1999) Printing small molecules as microarrays and detecting protein-ligand interactions en masses. *J. Am. Chem. Soc.* **121**, 7967–7968
2. Hergenrother, P. J., Depew, K. M., Schreiber, S. L. (2000) Small molecule microarrays: covalent attachment and screening of alcohol-containing small molecules on glass slides. *J. Am. Chem. Soc.* **122**, 7849–7850
3. Barnes-Seeman, D., Park, S. B., Koehler, A. N., Schreiber, S. L. (2003) Expanding the functional group compatibility of small-molecule microarrays: discovery of novel calmodulin ligands. *Angew. Chem. Int. Ed.* **42**, 2376–2379
4. Houseman, B. T., Mrksich, M. (2002) Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. *Chem. Biol.* **9**, 443–454
5. Kohn, M., Wacker, R., Peters, C., Schroder, H., Soulere, L., Breinbauer, R., Niemeyer, C. M., Waldmann, H. (2003) Staudinger ligation: a new immobilization strategy for the preparation of small molecule arrays. *Angew. Chem. Int. Ed.* **42**, 5830–5834
6. Lee, M.-r., Shin, I. (2005) Fabrication of chemical microarrays by efficient immobilization of hydrazide-linked substances on epoxide-coated glass surfaces. *Angew. Chem. Int. Ed.* **44**, 2881–2884
7. Lee, M.-r., Shin, I. (2005) Facile preparation of carbohydrate microarrays by efficient, covalent immobilization of unmodified carbohydrates on hydrazide-coated glass slides. *Org. Lett.* **7**, 4269–4272
8. Lesaicherre, M.-L., Lue, R. Y. P., Chen, G. Y. J., Zhu, Q., Yao, S. Q. (2002) Intein-mediated biotinylation of proteins and its application in a protein microarray. *J. Am. Chem. Soc.* **124**, 8768–8769
9. Kanoh, N., Kumashiro, S., Simizu, S., Kondoh, Y., Hatakeyama, S., Tashiro, H., Osada, H. (2003) Immobilization of natural products on glass slides by using a photoaffinity reaction and the detection of protein-small-molecule interactions. *Angew. Chem. Int. Ed.* **42**, 5584–5587
10. Bradner, J. E., McPherson, O. M., Mazitscheck, R., Barnes-Seeman, D., Shen, J. P., Dhaliwal, J., Stevenson, K. E., Duffner, J. L., Park, S. B., Neuberg, D. S., Nghiem, P., Schreiber, S. L., and Koehler, A. N. (2006) A robust small-molecule microarray platform for screening cell lysates. *Chem. Biol.* **13**, 493–504
11. Bradner, J. E., McPherson, O. M., and Koehler, A. N. (2006) A method for the covalent capture and screening of diverse small molecules in a microarray format. *Nat. Protocols* **1**, 2344–2352

Chapter 4

Detecting Protein–Small Molecule Interactions Using Fluorous Small-Molecule Microarrays

Arturo J. Vegas and Angela N. Koehler

Abstract

General binding assays involving microarrays of small molecules can be used to identify small molecule ligands for nearly any protein, even in the absence of knowledge about protein structure or function. Several suitable methods for manufacturing small molecule microarrays (SMMs) exist and different immobilization methods may be more or less preferable for any given application. Here, we describe a protocol for noncovalent and homogenous capture of small molecules using fluorous interactions between small molecules containing fluorocarbon tags and fluorocarbon-coated glass surfaces. These arrays are especially useful for applications that require display of compounds in a specific orientation such as screening biased libraries.

Key words: Small molecule microarrays, Fluorous, Noncovalent attachment, Homogenous display, HDAC, SAHA, Structure-binding relationships

1. Introduction

Small molecule microarrays (SMMs) are an attractive technology for the high-throughput identification of small molecule ligands for proteins. Thousands of compounds can be queried for their ability to bind a protein of interest in a single microarray. This approach has been used successfully to identify functional probes of extracellular growth factors (1), various enzymes (2–4), transcription factors (5), as well as pathogenic intron RNA (6). Various surface chemistries have been developed to covalently attach molecules onto solid substrates such as glass or silicon and have been reviewed elsewhere (7–9). Most of these approaches take advantage of either latent functionalities or specific appendages on the small molecules that react with the modified surface.

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_4,

These approaches can result in either a homogenous (4, 10) or a heterogenous (11, 12) display of small molecules and often require significant surface preparation. Perfluoroalkyl tags, otherwise known as fluorous tags, are versatile protecting groups for chemical synthesis of small molecule collections (13–15). These tags can facilitate noncovalent immobilization on fluorinated glass surfaces via a fluorous solvophobic effect (Fig. 1). This dual

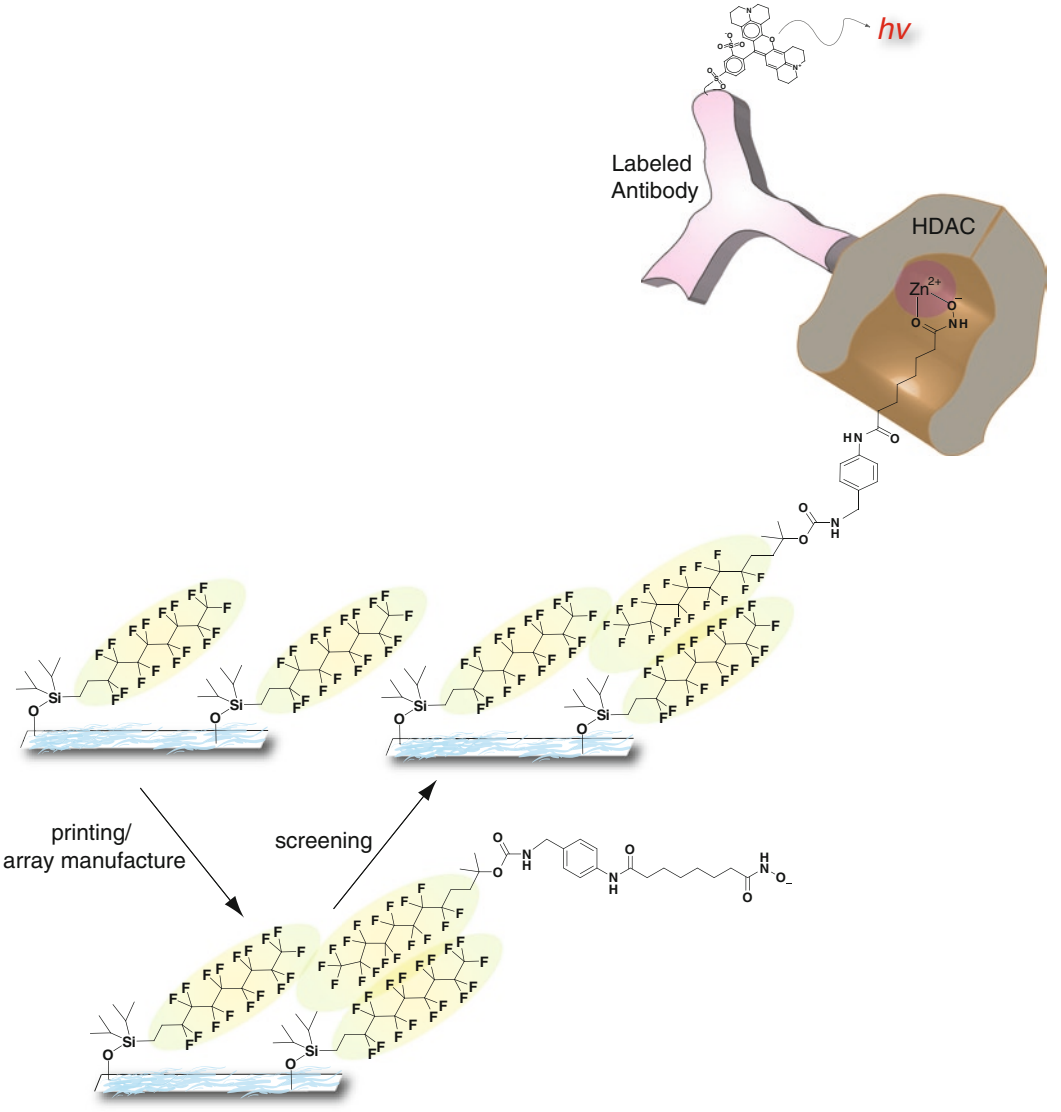


Fig. 1. Fluorous small molecule microarrays (FSMMs) allow for a homogenous display of small molecule functionality. Arrays are manufactured by noncovalent immobilization of fluorous-modified small molecules on a fluorous-modified glass surface (see Subheading 3.1). Incubation of arrays with purified protein (see Subheading 3.2) or lysates (see Subheading 3.3) followed by antibody detection reveals leads for protein ligands.

application of fluorous technology presents an integrated platform toward the synthesis and screening of small molecule collections. Pohl and coworkers originally demonstrated that fluorous microarrays can serve as a robust screening tool for identifying interactions involving proteins and unprotected carbohydrates (16–18). More recently, this approach has been extended to hydrophobic, drug-like small molecules (19, 20). Microarrays manufactured using this approach have low uniform background, excellent signal-to-noise, and consistent spot morphologies.

In this chapter, we present a step-by-step protocol for the manufacture of fluorous-based SMMs (FSMMs) that can be used to screen for protein–small molecule interactions. Specifically, we present a method using FSMMs to identify small molecules that bind and inhibit histone deacetylases (HDACs). HDACs catalyze the hydrolysis of *N*-acetyl groups on lysine residues found in the N-terminal tails of histone proteins (21). Reversible acetylation plays an important role in defining chromatin states and in regulating transcription from genomic DNA differentially across distinct tissues, thereby mediating cell differentiation, epigenetic inheritance, and various disease-related processes (22, 23). Identifying HDAC inhibitors though chemical screening is an increasingly active area of research (20, 24, 25). Compounds that resemble suberoylaniline hydroxamic acid (SAHA), a compound that inhibits multiple members of the HDAC family and that has been approved for the treatment of cutaneous T-cell lymphoma (26), were prepared with fluorous tags, printed, and screened in an effort to identify structure-binding relationships (20). The FSMMs allow for controlled molecular display of a critical chelator functionality that is required for enzyme inhibition. Protocols for screening a purified HDAC3/NCoR2 protein complex and HDAC3 residing in cellular lysates are described.

2. Materials

2.1. Compound Printing/Array Manufacture

1. Fluorous-functionalized glass slides (catalog #850-9100, Fluorous Technologies, Inc.).
2. OmniGrid®100 Microarrayer (Genomic Solutions) outfitted with an ArrayIt™ Stealth 48 pin printhead and SMP3 spotting pins (TeleChem International, Inc.), or equivalent.
3. Solutions of small molecules (~2.5–10 mM in DMSO) in 384-well V-bottom polypropylene plates (Abgene) (Fig. 2). Typical purity requirements for compounds are ≥90%.
4. Dimethylformamide (Sigma-Aldrich).

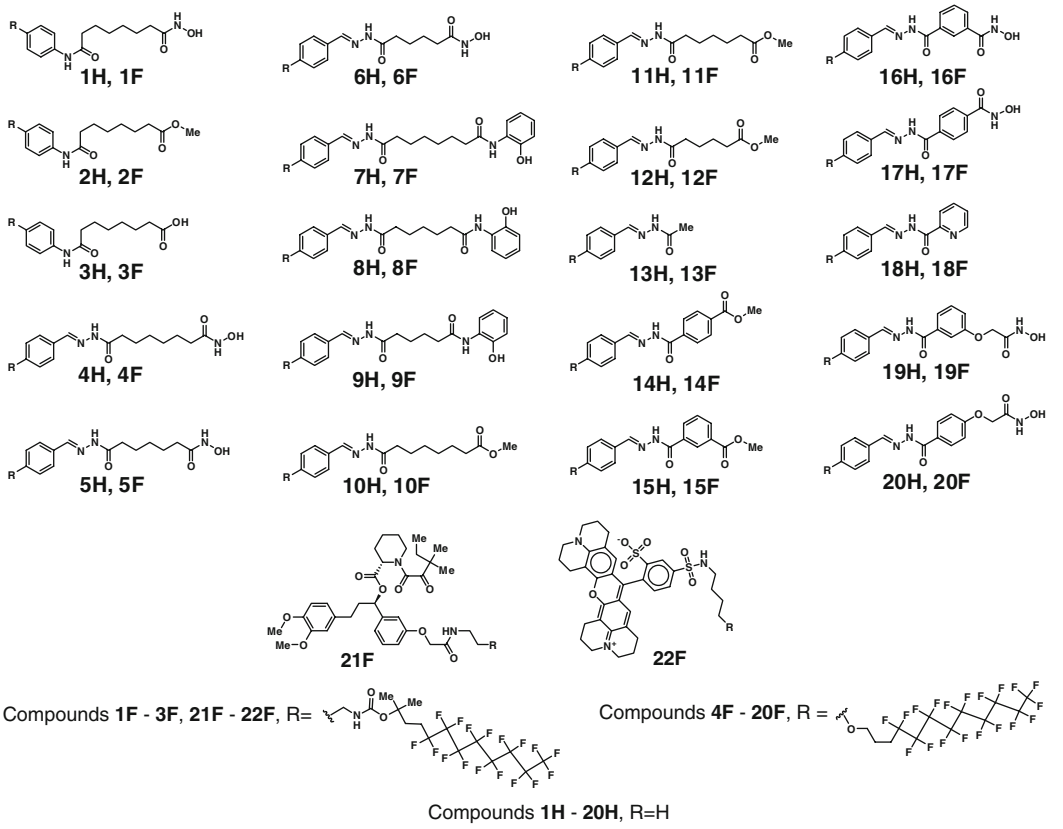


Fig. 2. Compounds printed onto fluorous-coated glass slides and tested as potential HDAC ligands. The compounds resemble suberoylaniline hydroxamic acid (SAHA), which inhibits multiple members of the HDAC family of enzymes. Nonfluorous analogs were used in secondary assay validation of FSMM positives. Compound 21F is a ligand for an unrelated protein (FKBP12) and serves as a negative control. Compound 22F is a Texas Red fluorescent dye used to frame array borders.

2.2. Screens with Pure Proteins

1. His-tagged HDAC3 was purchased in a complex with a GST-tagged NCoR2 peptide. This complex resulted in improved enzyme competence in all assays tested (BPS Biosciences).
2. HDAC assay buffer: 50 mM HEPES pH 7.4, 100 mM KCl, 0.005% Tween 20.
3. 4-well rectangular dish, polystyrene, sterile catalog #: 267061 (Nunc).
4. Incubating Rocking Platform Shaker (VWR).
5. Alexa Fluor® 647 anti-pentaHis antibody (Qiagen).
6. Distilled water (or Millipore-purified).
7. Axon 4000B or Axon 4200A scanner.
8. GenePix Pro 6.0 software (Axon Instruments, Inc.).

2.3. Screens with Lysates

1. 293-MSR cells.
2. T175 flasks (Nunc).
3. Dulbecco's Modified Eagle's Medium (DMEM, Gibco/BRL) supplemented with 10% fetal bovine serum (FBS, HyClone) and 0.6 mg/mL G418 (Invitrogen).
4. Sterile incubator.
5. Trypsin solution (1× in DMEM, Invitrogen).
6. Eppendorf compact centrifuge (VWR).
7. MIPP lysis buffer: 20 mM NaH_2PO_4, pH 7.2, 1 mM Na_3VO_4, 5 mM NaF, 25 mM β-glycerophosphate, 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 0.5% (v/v) Triton X-100.
8. Protease inhibitor cocktail (Sigma).
9. Parafilm (VWR).
10. Mouse anti-HDAC3 monoclonal primary antibody (Upstate).
11. Alexa Fluor® 647 goat anti-mouse polyclonal secondary antibody.
12. HDAC assay buffer: 50 mM HEPES pH 7.4, 100 mM KCl, 0.005% Tween.

3. Methods

Here, we describe a protocol for identifying protein–small molecule interactions using FSMMs. In this specific example, compounds resembling SAHA were printed and screened on the arrays in an effort to study structure-binding relationships. Fluorous technology facilitates the synthesis and purification of compounds as well as homogenous display within the microarray so that the chelating element, critical for recognition by the HDAC enzymes, is oriented away from the surface. Other immobilization strategies used for SMM manufacture might obscure the critical chelating elements through reaction with the modified glass slide (7, 11). As with all SMM assay platforms, primary assay positives should be confirmed using either secondary binding assays (e.g., surface plasmon resonance or isothermal titration calorimetry assays) and in functional assays (e.g., enzymatic or phenotypic) (8). In this study, nonfluorous analogs of each compound (*1H–20H*) were evaluated in HDAC3 enzyme inhibition assays (Fig. 5a) and surface plasmon resonance secondary binding assays (Fig. 5b). Results from both the FSMM and biochemical assays were congruent, with eight out of nine enzyme inhibitors scoring as positives in the array-based assay (20). Binding profiles generated using SMMs against different HDAC homologs should aid in the discovery of new selective inhibitors, which remains a challenge in chromatin research.

3.1. Compound Printing/Array Manufacture

1. Prepare stock solutions of compounds for printing by dissolving them in DMSO. Typical stocks are prepared in a concentration range of 2.5–10 mM depending upon solubility. Stock solutions are stored at –20°C.
2. Transfer 5 µl of each compound to a V-bottom 384-well polypropylene plate.
3. Clean fluorous-functionalized glass slides prior printing by using a gentle stream of air to remove any particulates from the surface (see Note 1).
4. Load slides carefully onto the slide platform or platen of an OmniGrid®100 Microarrayer (or equivalent), making sure that all slides are in the same orientation.
5. Prior to printing, clean the SMP3 spotting pins by sonicating them in dimethylformamide for 30 min. Note that washing protocols are critical to clean away hydrophobic compounds, especially dyes, and preventing the carryover of samples.
6. Load the clean SMP3 spotting pins onto the ArrayIt™ Stealth 48 pin printhead being careful to avoid touching the tips of the pins.
7. Design the printing configuration using the OmniGrid®100 software. Array features typically display diameters of 80–150 µm with 300 µm center-to-center spacing.
8. To ensure that compound solutions reside at the bottom of each well, centrifuge all compound stock plates at 400 × *g* for 1 min at 24.0°C using a Genevac HT-24 or standard benchtop centrifuge with microplate adapters.
9. Insert a clean glass blot pad and compound plates into the microplate positions on the array deck. Make sure that the compound plate is in the proper orientation with respect to well A1.
10. Print compounds in the desired array format. Instruct the microarrayer to spot 30 features on the blot pad with a 400 µm spacing center-to-center spacing between sample pick-up and actual printing onto fluorous slides. Within the print run, wash the pins five times with a 6 s incubation in dimethylformamide followed by vacuum drying in between each sample pick-up and deposition. Note that washing protocols are critical to clean away hydrophobic compounds, especially dyes, and preventing the carryover of samples (see Notes 2 and 3).
11. Once the print run has completed, allow slides to dry for at least 10 min prior to removing from platen.
12. Dried slides are packaged into 5-slide plastic boxes and stored in a –20°C freezer.

13. Quality control studies for each print run involved scanning arrays prior to screening and looking for the presence or absence of various fluorescent control features as well as screens to detect a known protein–ligand interaction involving compound *21F* and FKBP12 using conditions reported previously (11).

3.2. Screens with Pure Proteins

1. Purified HDAC3/NCoR2 was diluted to a protein concentration of 1 μg/mL in HDAC assay buffer.
2. Printed slides were incubated with purified HDAC3/NCoR2 (printed side facing *up*) in a 4-well dish. Each slide required approximately 7 mL of protein solution to fully cover the fluorous surface (see Note 4).
3. When performing a competition assay, add SAHA (compound *1H*) to a final concentration of 35 nM to the protein solution.
4. Gently agitate slides on a VWR incubating rocking platform shaker (speed value 15, tilt value 6) at room temperature for 30 min.
5. Discard the protein solution, and then immediately incubate slides with Alexa Fluor® 647 conjugated anti-pentaHis antibody (Qiagen) diluted 1:1,000 in HDAC assay buffer (7 mL per slide). This antibody is used to detect the His-tagged HDAC3 portion of the complex.
6. Gently agitate slides with antibody at room temperature for 30 min, as described previously.
7. Discard the antibody solution and quickly rinse slides (<10 s) with HDAC assay buffer followed by a quick rinse with purified distilled water.
8. Dry slides by centrifugation for 15 s. Immediately scan slides for fluorescence using an Axon 4000B or Axon 4200A scanner at 635 nm to detect binding of the Alexa Fluor® 647 labeled antibody (Fig. 3).
9. To analyze the array features, total fluorescence intensity values are calculated using a set 160 μm diameter circle centered over each feature using GenePix Pro 6.0 software (see Notes 6 and 7).

3.3. Screens with Lysates

1. Culture 293-MSR cells in a T175 flask to 90% confluency in 30 mL of DMEM medium supplemented with 10% fetal bovine serum and 0.6 mg/mL G418 at 37°C in a sterile incubator, ideally yielding five to ten million cells.
2. Aspirate the medium and treat cells with 4 mL of trypsin (1× in DMEM) for 5 min at 37°C.

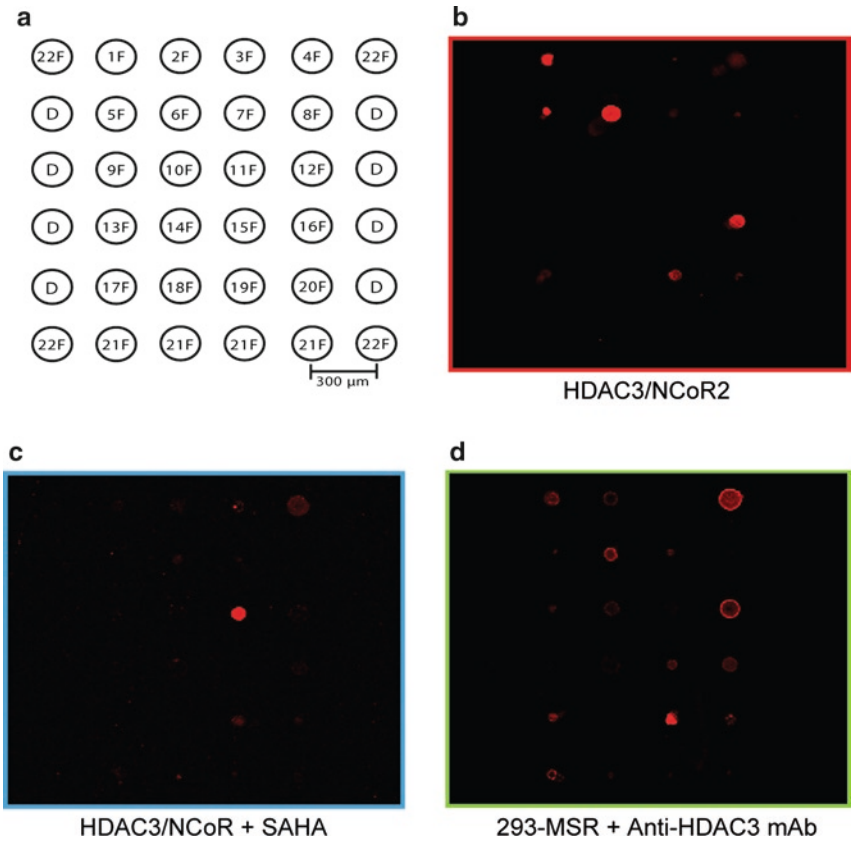


Fig. 3. (**a**) Array format of fluorous-modified compounds shown in Fig. 2 and (**b**) a representative scanned array after incubation with HDAC3/NCoR2 and Alexa Fluor® 647 labeled Anti-His antibody (635 nm). DMSO controls are designated as "D". (**c**) Representative array for a competition assay involving coincubation with 35 nM SAHA, a known HDAC inhibitor that binds at the enzyme active site. (**d**) Clarified 293-MSR lysate incubated with arrays, followed by incubation with a monoclonal anti-HDAC3 antibody and an Alexa Fluor® 647 labeled secondary antibody.

3. Dilute the cells with 20 mL of medium and transfer the solution to 50 mL falcon tube.
4. Form a cell pellet by spinning at 200 × *g* for 5 min. To remove residual trypsin, aspirate medium and gently resuspend cells with 20 mL of fresh medium.
5. Centrifuge again to reform cell pellet. Aspirate medium. Cell pellet can either be carried forward for an experiment, or be flash-frozen in a dry-ice/isopropanol bath and stored at −80°C for later use.
6. Prepare a clarified lysate by suspending cell pellet in 2.5 mL of MIPP lysis buffer with a 1:200 dilution of protease inhibitor cocktail for 10 min on ice. One cell pellet gives enough lysate for three microarray experiments.
7. Dispense 800 µL of solution into three 1.5 mL centrifuge tubes followed by centrifugation at 20,800 × *g* for 15 min at 4°C.

8. Dispense 600 μL of a clarified lysate droplet (avoid disturbing the cell pellet) on a clean sheet of parafilm (see Note 4).
9. Incubate one small molecule microarray with the lysate droplet by gently placing the slide printed face down on the droplet. Optimal spreading of the solution is achieved by first allowing droplet to touch the edge of the slide and then slowly lowering the rest of the slide on the solution. Avoid air bubbles (see Note 5).
10. Incubate solution with slide for 30 min at room temperature.
11. During the lysate incubation, prepare 700 μL of antibody solution by mixing anti-HDAC3 monoclonal primary antibody and Alexa Fluor® 647 goat anti-mouse polyclonal secondary antibody together at a ratio of 1:1 and at a dilution of 1:1,000 in HDAC assay buffer.
12. Prepare a 600 μL droplet of antibody mixture on parafilm as described above.
13. Once lysate incubation is complete, quickly transfer and place slide face down on antibody droplet, assuring optimal spreading as before. Incubate at room temperature for 30 min.
14. Quickly rinse slide with HDAC assay buffer (< 10 s), and then briefly rinse in distilled water. Dry slide by centrifugation and scan slide immediately on an Axon 4000B or Axon 4200A scanner at 635 nm to detect binding (see Notes 6 and 7). Typical results obtained are depicted in Figs. 4 and 5.

4. Notes

1. Dust particles can confound the analysis of microarrays due to autofluorescence. A gentle stream of air can be used to remove dust particles from the slide surface prior to printing.
2. Occasionally, we note that compound stock solutions carryover from one sample pick-up to the next. When this happens, we increase the duration of the pin wash cycle or increase the number of wash cycles in between each sample pick-up. If a blot pad is used for the print run, we have to be careful in cleaning it with methanol and bibulous paper after every 768 samples.
3. Some dyes can create problems with sample carryover if the pins are not washed sufficiently or if the sample concentration is high. We typically print dyes at lower concentrations (2.5 mM) than our standard sample concentration of 10 mM. We have also noticed that spot morphologies are more consistent with dyes printed at 2.5 mM.

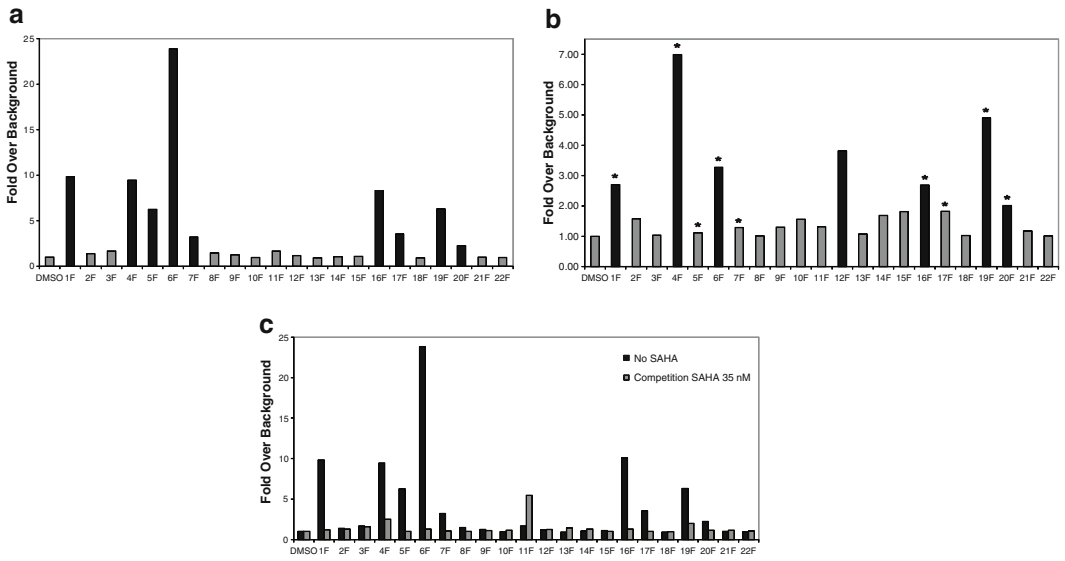


Fig. 4. Arrays are analyzed by averaging fluorescence intensity over experimental replicates and by normalizing against averaged DMSO fluorescence intensity (the background) across the array. (**a**) Purified HDAC3/NCoR2 analysis with positive enzyme binders classified by greater than twofold above the background and indicated by *black bars*. (**b**) Lysate HDAC3 analysis with positive enzyme binders classified by greater than twofold above the background and indicated by *black bars*. *Black asterisks* indicate compounds that displayed greater than twofold above background with purified HDAC3/NCoR2. (**c**) Competition assay analysis comparing fold over background with purified HDAC3/NCoR2 with and without the addition of SAHA at 35 nM.

4. In our experience, roughly 7 mL of aqueous solution is required to fully immerse a fluorous-coated slide in a dish, whereas less hydrophobic microarray slides require half the volume. If a sample is precious and there is less material, we suggest adopting a protocol similar to that used to screen lysates (see Subheading 3.3, steps 8–14), where the slide is placed printed face down onto a 600 µL sample droplet held on parafilm.
5. Be careful to avoid air bubbles in the protein solution under the slide when using the droplet protocol described in Subheading 3.3. This can reduce reproducibility among replicate slides as the protein does not consistently come in contact with the array surface.
6. If features appear white during scanning, you have reached the saturation limit of the scanner. Lower the photomultiplier tube (PMT) voltage and scan again.
7. Several problems may give rise to missing printed features. First, stock solution may not have been properly delivered to the slide surface. It is critical that the stock solutions reside in the bottom of the plate wells and not on the sides

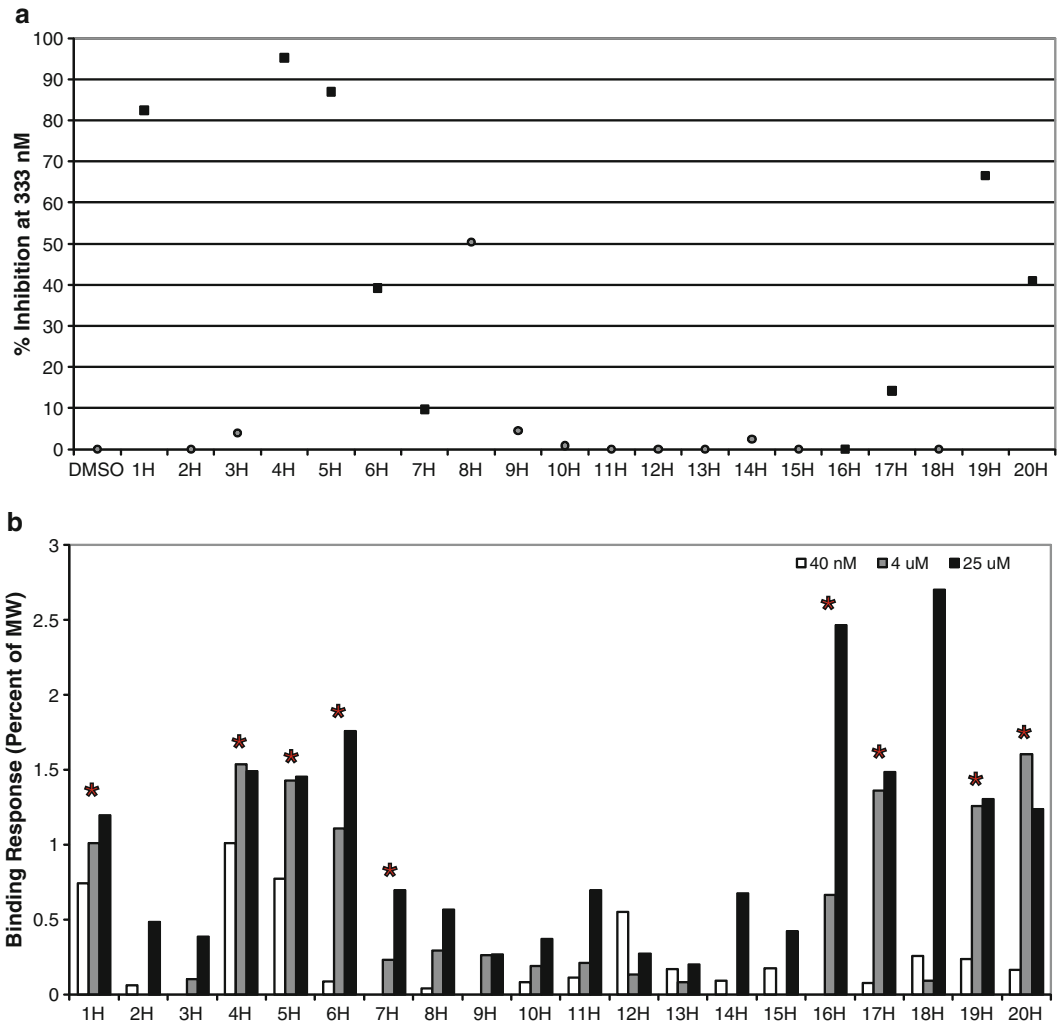


Fig. 5. Secondary assay validation of microarray positives using non-fluorous compound analogs in (**a**) HDAC enzymatic activity assay data shown as percent inhibition at 333 nM and (**b**) SPR ranking assay at 40 nM, 4 μM, and 25 μM. *Black squares* and *black asterisks* indicate compounds with fluorous analogs that are classified as positives in microarray analysis.

of the well. It is also critical that the *z*-axis is aligned so that the pins make contact with the solution in each well. We also physically inspect each plate prior to printing to be sure that samples have not evaporated, especially in edge wells. We also inspect the arrayer printhead to be sure that all pins move freely. Pins must also be inspected under a microscope to make sure that the tip is not obstructed, bent, or corroded.

Acknowledgments

The authors would like to thank Professor Stuart Schreiber, Dr. James Bradner, Dr. Weiping Tang, Olivia McPherson, Edward F. Greenberg, Dr. Ralph Mazitschek, Dr. Carlos Tassa, Dr. Marvin Yu, and Dr. Philip Yeske for providing materials, technical support, or advice that was relevant to developing this protocol. Work described herein was funded in whole or in part with Federal funds from the National Cancer Institute's Initiative for Chemical Genetics, National Institutes of Health, under contract no. N01-CO-12400.

References

1. Stanton, B. Z., Peng, L. F., Maloof, N., Nakai, K., Wang, X., Duffner, J. L., Taveras, K. M., Hyman, J. M., Lee, S. W., Koehler, A. N., Chen, J. K., Fox, J. L., Mandinova, A., and Schreiber, S. L. (2009) A small molecule that binds Hedgehog and blocks its signaling in human cells, *Nat Chem Biol 5*, 154–156.
2. Kwon, S. J., Lee, M. Y., Ku, B., Sherman, D. H., and Dordick, J. S. (2007) High-throughput, microarray-based synthesis of natural product analogues via in vitro metabolic pathway construction, *ACS Chem Biol 2*, 419–425.
3. Uttamchandani, M., Lee, W. L., Wang, J., and Yao, S. Q. (2007) Quantitative inhibitor fingerprinting of metalloproteases using small molecule microarrays, *J Am Chem Soc 129*, 13110–13117.
4. Urbina, H. D., Debaene, F., Jost, B., Bole-Feysot, C., Mason, D. E., Kuzmic, P., Harris, J. L., and Winssinger, N. (2006) Self-assembled small-molecule microarrays for protease screening and profiling, *Chembiochem 7*, 1790–1797.
5. Koehler, A. N., Shamji, A. F., and Schreiber, S. L. (2003) Discovery of an inhibitor of a transcription factor using small molecule microarrays and diversity-oriented synthesis, *J Am Chem Soc 125*, 8420–8421.
6. Labuda, L. P., Pushechnikov, A., and Disney, M. D. (2009) Small molecule microarrays of RNA-focused peptoids help identify inhibitors of a pathogenic group I intron, *ACS Chem Biol 4*, 299–307.
7. Vegas, A. J., Fuller, J. H., and Koehler, A. N. (2008) Small-molecule microarrays as tools in ligand discovery, *Chem Soc Rev 37*, 1385–1394.
8. Duffner, J. L., Clemons, P. A., and Koehler, A. N. (2007) A pipeline for ligand discovery using small-molecule microarrays, *Curr Opin Chem Biol 11*, 74–82.
9. Uttamchandani, M., Wang, J., and Yao, S. Q. (2006) Protein and small molecule microarrays: powerful tools for high-throughput proteomics, *Mol Biosyst 2*, 58–68.
10. Kohn, M., Wacker, R., Peters, C., Schroder, H., Soulere, L., Breinbauer, R., Niemeyer, C. M., and Waldmann, H. (2003) Staudinger ligation: a new immobilization strategy for the preparation of small-molecule arrays, *Angew Chem Int Ed Engl 42*, 5830–5834.
11. Bradner, J. E., McPherson, O. M., Mazitschek, R., Barnes-Seeman, D., Shen, J. P., Dhaliwal, J., Stevenson, K. E., Duffner, J. L., Park, S. B., Neuberg, D. S., Nghiem, P., Schreiber, S. L., and Koehler, A. N. (2006) A robust small-molecule microarray platform for screening cell lysates, *Chem Biol 13*, 493–504.
12. Miyazaki, I., Okumura, H., Simizu, S., Takahashi, Y., Kanoh, N., Muraoka, Y., Nonomura, Y., and Osada, H. (2009) Structure-affinity relationship study of bleomycins and Shble protein by use of a chemical array, *Chembiochem 10*, 845–852.
13. Studer, A., Hadida, S., Ferritto, R., Kim, S. Y., Jeger, P., Wipf, P., and Curran, D. P. (1997) Fluorous synthesis: a fluorous-phase strategy for improving separation efficiency in organic synthesis, *Science 275*, 823–826.
14. Zhang, W., and Lu, Y. (2007) 96-well plate-to-plate gravity fluorous solid-phase extraction (F-SPE) for solution-phase library purification, *J Comb Chem 9*, 836–843.
15. Curran, D. P. (2008) Chemistry. Fluorous tags unstick messy chemical biology problems, *Science 321*, 1645–1646.

16. Ko, K. S., Jaipuri, F. A., and Pohl, N. L. (2005) Fluorous-based carbohydrate microarrays, *J Am Chem Soc 127*, 13162–13163.
17. Jaipuri, F. A., Collet, B. Y., and Pohl, N. L. (2008) Synthesis and quantitative evaluation of Glycero-D-manno-heptose binding to concanavalin A by fluorous-tag assistance, *Angew Chem Int Ed Engl 47*, 1707–1710.
18. Chen, G. S., and Pohl, N. L. (2008) Synthesis of fluorous tags for incorporation of reducing sugars into a quantitative microarray platform, *Org Lett 10*, 785–788.
19. Nicholson, R. L., Ladlow, M. L., and Spring, D. R. (2007) Fluorous tagged small molecule microarrays, *Chem Commun (Camb) 14*, 3906–3908.
20. Vegas, A. J., Bradner, J. E., Tang, W., McPherson, O. M., Greenberg, E. F., Koehler, A. N., and Schreiber, S. L. (2007) Fluorous-based small-molecule microarrays for the discovery of histone deacetylase inhibitors, *Angew Chem Int Ed Engl 46*, 7960–7964.
21. Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996) A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p, *Science 272*, 408–411.
22. Botrugno, O. A., Santoro, F., and Minucci, S. (2009) Histone deacetylase inhibitors as a new weapon in the arsenal of differentiation therapies of cancer, *Cancer Lett 280*, 134–144.
23. Kouzarides, T. (2007) Chromatin modifications and their function, *Cell 128*, 693–705.
24. Wong, J. C., Hong, R., and Schreiber, S. L. (2003) Structural biasing elements for in-cell histone deacetylase paralog selectivity, *J Am Chem Soc 125*, 5586–5587.
25. Khan, N., Jeffers, M., Kumar, S., Hackett, C., Boldog, F., Khramtsov, N., Qian, X., Mills, E., Berghs, S. C., Carey, N., Finn, P. W., Collins, L. S., Tumber, A., Ritchie, J. W., Jensen, P. B., Lichenstein, H. S., and Sehested, M. (2008) Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors, *Biochem J 409*, 581–589.
26. Kelly, W. K., Richon, V. M., O'Connor, O., Curley, T., MacGregor-Curtelli, B., Tong, W., Klang, M., Schwartz, L., Richardson, S., Rosa, E., Drobnjak, M., Cordon-Cordo, C., Chiao, J. H., Rifkind, R., Marks, P. A., and Scher, H. (2003) Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously, *Clin Cancer Res 9*, 3578–3588.

Chapter 5

A Method for Small Molecule Microarray-Based Screening for the Rapid Discovery of Affinity-Based Probes

Haibin Shi, Mahesh Uttamchandani, and Shao Q. Yao

Abstract

We describe herein a new method for the high-throughput identification of affinity-based probes (A*f*BPs) using a small molecule microarray (SMM) approach. A hydroxylethylene-based small molecule library was first generated by solid-phase combinatorial synthesis. The library was tagged with biotin to facilitate immobilization on avidin-coated slides. Preliminary screening with γ-secretase (both the recombinantly purified protein as well as cellular lysates overexpressing the enzyme) was carried out, in order to identify potential small molecule binders, which were subsequently redesigned into A*f*BPs. Several specific and potent probes for γ-secretase were thus identified through the binding profiles observed on the SMMs. The SMM platform was able to sensitively and conveniently report activity-based binding interactions between aspartic proteases and their small molecule inhibitors. This new approach thus provides a potentially more rapid and efficient method for developing A*f*BPs using SMMs.

Key words: Small molecule microarrays, Affinity-based probes, γ-secretase, Solid-phase synthesis, Hydroxylethylene scaffolds

1. Introduction

Small molecule microarrays (SMMs) provide a valuable platform for the large-scale, quantitative determination of protein–ligand interactions in high-throughput (1). It offers a cheap and convenient method for the screening of thousands of compounds rapidly and has been successfully used in ligand identification (2), and in protein profiling (3). Furthermore, microarray-based technologies have also been used in the discovery of biosensors and biomarkers for disease diagnostics (4). We describe herein an SMM approach for the high-throughput identification of affinity-based probes (A*f*BPs), using γ-secretase as our enzyme target. This strategy may be further applied to the identification

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_5, © Springer Science+Business Media, LLC 2010

of affinity-based probes for aspartic protease, as well as other classes of enzymes.

Aspartic proteases are among the smallest groups of proteases, with only 15 members identified in the human genome. These enzymes have nevertheless proven to be a rich source for drug targets for the pharmaceutical industry (5). There remains, however, a gap in the functional annotation, as well as in the structural information available, for members of this group. It is thus a challenge to profile the activity of aspartic proteases, especially with regard to understanding their physiological function as well as in the development of therapeutics. To address this need, we have developed an SMM-based approach that facilitates the development of A*f*BPs for enzymes. These arrays may be applied for screening the ligands of enzymes, which are either recombinantly expressed and purified or overexpressed in cell lysates. The ligands identified are thereafter converted into specific probes for enzyme profiling. Our approach is described here using γ-secretase as a model.

The overall strategy is illustrated in Fig. 1. A hydroxylethylene-based small molecule inhibitor library was generated using solid-phase combinatorial synthesis. The hydroxyethylene scaffold is a transition state analog of aspartic proteases and thus target the inhibitors to bind the active site of these enzymes. After incubating membrane fraction of γ-30 cell lysates with inhibitor library onto the microarrays, highly reproducible and distinct binding profiles were obtained against the 198-member library. Two compounds which showed the strongest relative binding fluorescence were directly converted to probes which contained a benzophenone (BP)

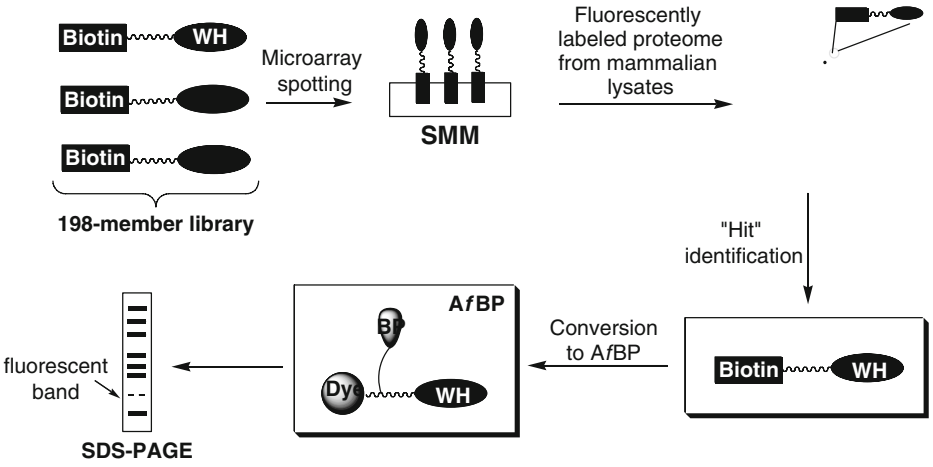


Fig. 1. Overall strategy of the small molecule microarray (SMM)-facilitated platform for high-throughput identification of A*f*BPs. A small molecule library was first immobilized on slide followed by screening with recombinant proteins or cellular lysates labeled with Cy3 dye. The stronger binders were then converted to affinity-based probes for applications in in vivo or in vitro protein profiling.

photo-crossing unit and a tertraethylrhodamine (TER) reporter or biotin handler according to our previous click chemistry strategy using the 1–3 dipolar cycloaddition reaction (6). To demonstrate the capability of the probes for proteomic profiling of γ-secretase in cellular lysates specifically, we carried out the in-gel fluorescence labeling experiments and pull down experiments. Our western blotting results established that the probe *F24* was able to specifically label N-terminal fragment of presenilin 1 (PS-NTF), the catalytic core of γ-secretase.

Traditional methods of probe design have relied on existing inhibitors of γ-secretase (7–10). Our approach facilitated the discovery of inhibitors of γ-secretase and facilitates the translation of these "hits" into probes for in vitro or in vivo profiling of the target enzymes. Our approach thus facilitates the high-throughput design and discovery of A*f*BPs for the functional profiling of aspartic proteases, as well as potentially other different classes of enzymes.

2. Materials

2.1. Preparation of Avidin Slides

1. Piranha solution (H_2SO_4:H_2O_2 = 7:3) (see Note 1).
2. MilliQ water.
3. Dimethylformamide (HPLC grade, Fischer Scientific).
4. Avidin (Pierce).
5. Succinic anhydride (Sigma-Aldrich).
6. *N*-hydroxysuccinimide (Sigma-Aldrich).
7. O-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU;GL Biochem).
8. *N*-hydroxybenzotriazole (HOBt; GL Biochem).
9. *N*-ethyldiisopropylamine (DIEA; Merck).
10. APTES solution: 380 mL 100 % ethanol, 8 mL H_2O and 12 mL aminopropyltriethoxisilane (Sigma-Aldrich).
11. Carboxylic acid activation solution: 400 mL of DMF (HPLC grade), 12 g succinic anhydride and 18 mL of 1 M $Na_2B_4O_7$ (pH 9).
12. NHS activation solution: 15 mL of DMF, 565 mg HBTU, 173 mg *N*-hydroxysuccinimide, 550 µL of DIEA.
13. Avidin solution: 2 mg avidin (for 30 slides), 1.5 mL of MilliQ water, 30 µL of 0.5 M $NaHCO_3$, pH 9.
14. Quenching solution: 2 mM aspartic acid in 0.5 M $NaHCO_3$ (pH 9).

2.2. Spotting

1. Small molecule library.
2. Phosphate buffered saline (PBS) (pH 7.4).
3. 384-well polypropylene microarray plate (Genetix).

2.3. Protein Labeling, Incubations, and Scanning

1. Cy3 or Cy5 *N*-hydroxysuccinimide ester (Amersham, GE Healthcare, USA).
2. Hydroxylamine (Sigma-Aldrich).
3. G-25 spin-column (Amersham, GE Healthcare, USA).
4. Sodium phosphate buffer (PBS) (pH 7.4).
5. Bovine serum albumin (BSA; Sigma-Aldrich).
6. Incubation buffer: 0.05% Tween 20 in PBS.

2.4. Synthesis of Probes

1. Alkyne and azide probe fragments.
2. *N,N*-Diisopropylethylamine (DIEA) (Sigma).
3. Copper sulfate ($CuSO_4 \cdot 5H_2O$) and sodium ascorbate (Sigma).
4. Solvents: Dimethylsulfoxide (DMSO; Fisher Scientific), distilled water (dH_2O).

2.5. Gel-Based Labeling and Pull-Down Experiments

1. γ-30 cell line.
2. Dulbecco's modified Eagle's medium (Sigma).
3. Fetal calf serum and 1% penicillin/streptomycin/puromycin/zeocine/geneticin/Hygromycin (Invitrogen).
4. Bradford protein assay (Bio-Rad).
5. 12% SDS-PAGE gels.
6. Blocking buffer: 2.5% (w/v) BSA in 0.05% Tween in PBS buffer.
7. PVDF membrane.
8. Antibody for PS1 (Santa Cruz Biotechnology).
9. Donkey anti-goat conjugated HRP (Santa Cruz Biotechnology).
10. Avidin-agarose beads (Thermo Scientific).
11. Neutravidin-conjugated HRP (Pierce).
12. SuperSignal West Pico kit (Pierce).
13. HEPES buffer: 10 mM HEPES, 1 mM EDTA, 150 mM NaCl, 10% Glycerol, pH 8.0.
14. Elution buffer: 200 mM Tris–HCl, 400 mM DTT, 8% SDS, pH 6.8.

2.6. Equipments

1. 75 mm by 25 mm glass slides (Sigma-Aldrich).
2. Metal slide rack and glass staining jar.

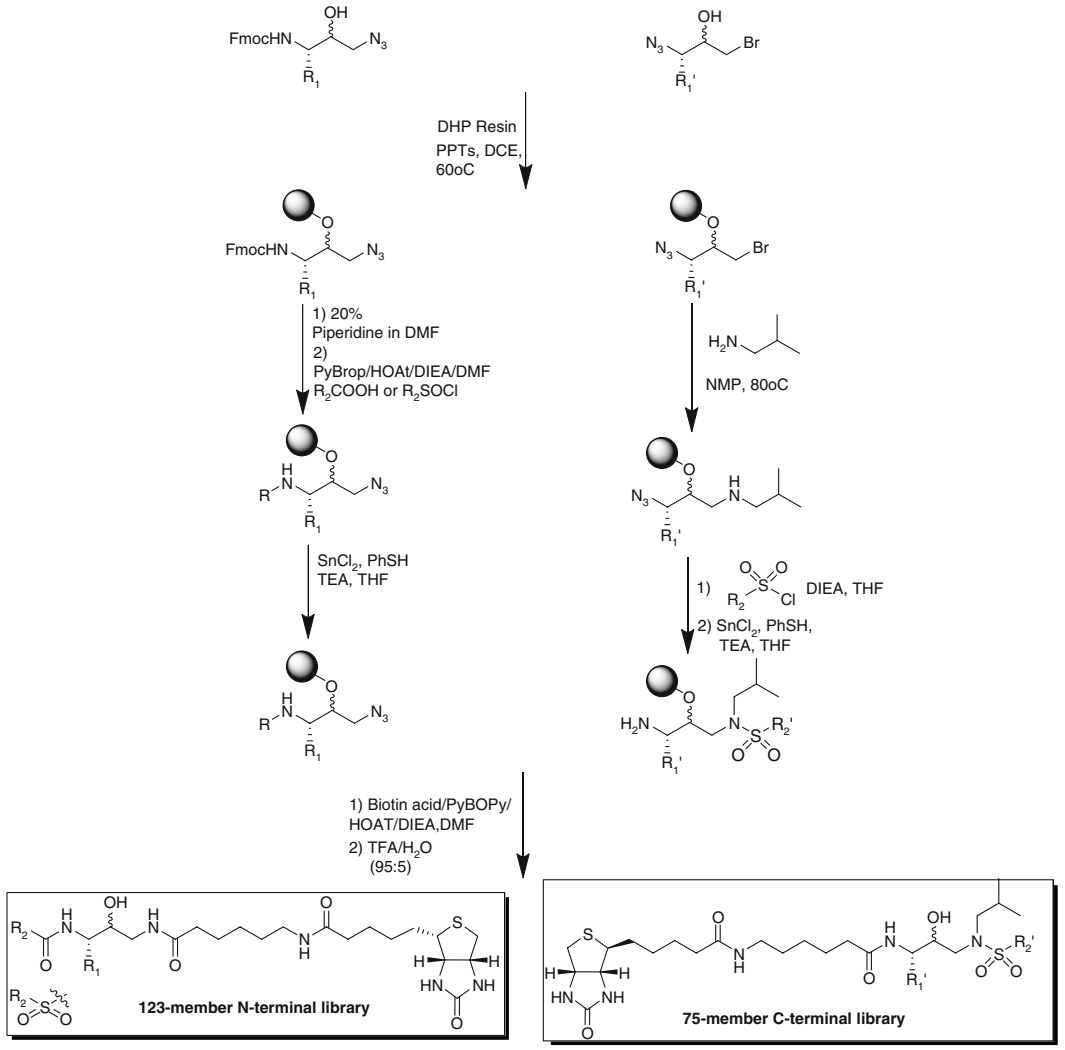
3. Magnetic stirring bars.
4. Fume cabinet.
5. Glass desiccators and a vacuum pump.
6. Oven (set to 150°C).
7. Arrayer (ESI SMA) fitted with Stealth Micro Spotting pins (Telechem International, cat. no. SMP10).
8. Coverslips (60 mm by 22 mm) or (40 mm by 20 mm).
9. ArrayWoRx Microarray Scanner (Applied Precision).
10. IRORI MicroKans.
11. IRORI radiofrequency tag.
12. Rotary evaporator (Buchi).
13. Vacuum evaporator (Genevac HT-4X).
14. Centrifuge (Hettich-Zentrifugen, model Rotina 35).
15. Shimadzu liquid chromatography-mass spectrometry-ion trap time-of-flight system equipped with autosampler (cat. no. LCMS-2010EV).
16. Typhoon 9200 fluorescence gel scanner (GE).

2.7. Software

1. ArrayWoRx Microarray Software (or equivalent).
2. Microsoft Excel (or equivalent).
3. Typhoon 9200 fluorescence image analysis software.

3. Methods

The 198-member library used in this study was synthesized by solid-phase combinatorial chemistry followed by immobilization onto avidin slides. After screening with pure protein or cellular lysates labeled with Cy3 dyes, the fluorescence intensity measured is indicative of the level of protein bound to the respective small molecule spots on the arrays. Based on the binding profile, several selective and potent ligands for γ-secretease were identified. All compounds were derived from a hydroxylethylene core group (see Scheme 1). In the 123-member N-terminal sublibrary the R_1 and R_2 positions correspond to the P_1 and P_2 residues. These were varied with aromatic/aliphatic moieties (R_1 = Phe, Leu, Ala; R_2 = aromatic/aliphatic acids). In the 75-member C-terminal sublibrary, the R_1 and R_2 positions correspond to the P_1 and $P_{1/2}$ positions, respectively, and were similarly varied. In both sublibraries, a biotin tag and a linker were introduced in each compound for subsequent immobilization onto SMM. All 198 compounds were analyzed by LC-MS, which confirmed the library members were pure (>80%) (11).



Scheme 1. Design and synthesis of the 198-member hydroxyethylene-based small molecule library. The library contained a biotin tag to facilitate immobilization onto avidin-coated glass slides.

3.1. Preparation of Avidin-Functionalized Glass Slides

1. Store glass slides in piranha solution until needed (see Note 1).
2. Remove slides required from piranha solution carefully and rinse ten times with MilliQ water to remove any trace of acid (see Note 2).
3. Rinse finally with 95% ethanol, and dry slides in fume hood.
4. Prepare APTES solution in a glass staining jar and stir for 10 min, with the glass lid covered (see Note 3).
5. Immerse the slide rack in this solution for 2 h with constant stirring.
6. Rinse slides with 95% ethanol four times.

7. Place the whole metal tray with the slides in a covered glass dish and cure in the 150°C oven for at least 2 h (see Note 4).
8. Cool down the slides before rinsing again with 95% ethanol. Flush the slides under a dry stream of nitrogen to remove excess ethanol and air dry slides in the fume hood.
9. Soak amine slides in the carboxylic acid activation solution for 20 min.
10. Thereafter, soak in a 95°C water bath for 2 min with constant shaking.
11. Rinse slides with 95% ethanol, flush with N_2, and then air dry in the fume hood.
12. Prepare the NHS activation solution.
13. Apply 450 μL of the NHS activation solution onto each carboxylic acid functionalized slide.
14. Cover with cover slips, and then incubate for 3 h at room temperature.
15. Rinse with 95% ethanol and air dry in the fume hood.
16. Prepare the avidin solution, apply 50 μL of the above mixture solution onto each slide and cover the slides with cover slips, and then incubate for 35 min at room temperature.
17. Rinse slides two times with MilliQ water.
18. Quench in a dish with quenching solution for 15 min.
19. Rinse with MilliQ water and transfer back onto metal tray, flush with N_2, and air dry in the fume hood.
20. Store slides in the fridge at 4°C until required for spotting, and rinse with water before use.

3.2. Spotting

1. Prepare 16 μL of each compound solution in a 384-well microtiter plate. The compounds were around 1 mM in PBS/DMSO (1:1) (see Note 5).
2. Spot the solutions (with at least duplicate spots for each compound) on avidin-functionalized glass slides.
3. The slides were allowed to stand for 1 h on the printer platform.
4. Store at 4°C until use.

3.3. Sample Preparation

1. Minimally label protein or lysate samples with either Cy3 or Cy5 *N*-hydroxysuccinimide ester for 1 h on ice in PBS.
2. Quench with tenfold molar excess of hydroxylamine for a further 1 h.
3. Remove the excess dye either by extensive dialysis at 4°C overnight or a sephadex G-25 spin-column.
4. Analyze by SDS-PAGE to ensure successful labeling of target proteins.

3.4. Incubation on Microarray

1. Rinse slides with PBS for 10 min and block with PBS-containing 1% BSA for 1 h.
2. Dilute labeled enzyme and lysate solution (to a final concentration of 1 μM) with HEPES buffer (1×) containing 0.1% Tween 20.
3. Apply 30 μL labeled protein or lysate samples to the slides under coverslip (40 mm by 20 mm) (see Note 6).
4. Incubate for 1 h at room temperature.
5. Rinse the slides briefly with water followed by washing with PBS + 0.05% Tween 20 for three times (10 min/time) with gentle shaking.
6. Scan using an ArrayWoRx microarray scanner installed with the relevant filters.

3.5. Scanning of the Slides

1. Scan slides on a microarray scanner equipped with the relevant filters (Cy3: $\lambda_{ex/em} = 548/595$ nm; Cy5: $\lambda_{ex/em} = 633/685$ nm).
2. Typical results obtained are displayed in Fig. 3a (screened against fluorescently labeled membrane fraction of γ-30 cell lysates). Of the strong binders, most were identified as members of the C-terminal sublibrary containing an alanine residue at the P_1 position. C-c-5 and C-c-24 showed the strongest relative fluorescence binding.

3.6. Synthesis of Probes for γ-secretease

Based on the microarray screening results, two stronger binders, C-c-5 and C-c-24, were identified and their corresponding A*f*BPs (both tetraethylrhodamine, TER, and biotinylated versions) were synthesized using "Click" Chemistry as shown in Fig. 2. A negative control probe TER-A31 (from the spot N-a-31 from the SMM screening which showed no binding and was deemed negative) was also synthesized.

1. Synthesize the TER-BP-alkyne and Biotin-BP-alkyne linkers (12).
2. Dissolve the alkyne (1.2 eq) and azide (1.0 eq; final concentration: 10.0 mM) fragments in a minimal amount of DMSO.
3. Add this to a final mixture of DMSO/water solution (1:1; 2 mL) and shake for a few minutes to obtain a clear solution.
4. Add catalytic amounts of $CuSO_4$ (0.1 eq) and sodium ascorbate (0.4 eq) and continue shaking at room temperature for another 12 h.
5. Analyze the reaction product with LC-MS to ensure complete consumption of the azide and quantitative formation of the triazole product in all cases.

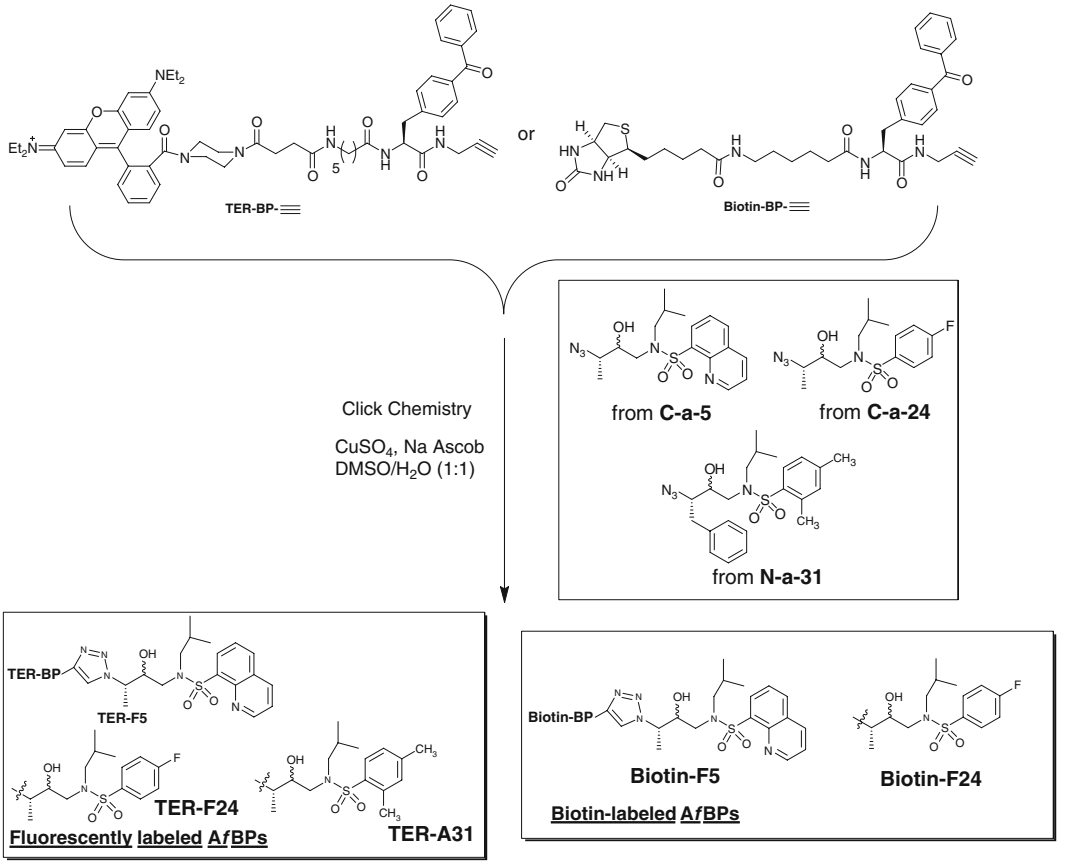


Fig. 2. "Click" assembly of the fluorescently labeled and biotin-containing affinity-based probes (A*f*BPs). A total of five probes were synthesized and obtained from the initial SMM screens.

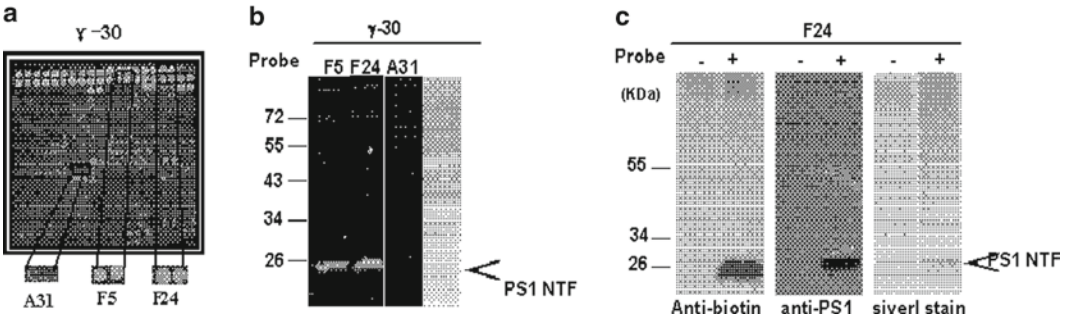


Fig. 3. (**a**) SMM of the 198-member library screened against fluorescently labeled membrane fraction of γ-30 cell lysates, with microarray image showing selected binders (F5 and F24) and a nonbinder (A31). All compounds were printed in duplicate; (**b**) In-gel fluorescence profiling of γ-30 lysates using the set of A*f*BPs designed using the "hits" identified using SMM (F5, F24); (**c**) Pull-down experiments from γ-30 lysates and western blotting identified the protein labeled with Biotin-*F24* as PS-NTF.

6. Purify the final probes that were subsequently purified by prep-HPLC and characterize/confirm the structure and purity by NMR and LC-MS.

3.7. Characterization of AfBPs with γ-30 Cell Lysates

In-gel fluorescence labeling and pull-down experiments were carried out with three TER-containing probes, TER-F5, TER-F24, and TER-A-31 (negative control) against mammalian cell lysates prepared from the γ-30 cell line.

1. Incubate 15 μg total lysates (determined by Bradford assay) with the probes (5 μM final concentration; 5% DMSO) in HEPES buffer for 30 min at RT.
2. After 30 min incubation, irradiate samples for 25 min using a B100A lamp (UVP) at a distance of 5 cm, on ice.
3. After irradiation, boil samples for 10 min with 4 μL of 6 × SDS loading buffer, resolved on a 12% SDS-PAGE followed by in-gel fluorescence scanning with a Typhoon 9200 gel scanner (for typical results obtained, see Fig. 3b).
4. For pull-down experiments, label 2 mg of lysates with 5 μM probe F24 in a 2-mL reaction using the above described conditions.
5. After labeling, precipitate the lysates in acetone and resolubilize in 0.1% SDS in PBS with brief sonication.
6. Incubate the resuspended sample with avidin-agarose beads at RT for 30 min.
7. After centrifugation, remove the supernatant and wash beads with 1% SDS in PBS for four times.
8. After washing, boil the beads that were in elution buffer.
9. Run the labeled samples on a 12% SDS PAGE gel.
10. After SDS-PAGE gels separation, transfer proteins that were onto a PVDF membrane and block the membranes with blocking buffer for 1 h.
11. Incubate membranes for 1 h at room temperature with neutravidin-conjugated HRP (diluted 1:10,000 in PBS) or anti-presenilin antibodies (diluted 1/5,000 in PBS) with shaking.
12. Wash with PBST for three times, and incubate with the appropriate secondary antibody for 1 h at room temperature with shaking.
13. After incubation, wash membrane again with PBST for three times.
14. Use a luminescence kit to develop the blot.
15. Typical western blot results obtained are shown in Fig. 3c.

4. Notes

1. To prepare the piranha solution, pour H_2SO_4 first and then add H_2O_2 slowly, make sure that the temperature does not increase too much since it can get extremely hot. The mixture H_2SO_4/H_2O_2 (piranha) solution is a strong oxidant. It reacts violently with organic materials. It can cause severe skin burns. It must be handled with extreme care in a well-ventilated fume hood while wearing appropriate chemical safety protection.
2. Use only powder-free gloves or bare hands to handle slides.
3. Shake the slide tray every once in a while to prevent small air bubbles accumulating on the slide surfaces. In the meantime, on the other hot plate start boiling deionized water (with a stirrer bar), until it reaches about 95°C.
4. Make sure that each slide is kept separate from each other. Cure for at least 2 h, but they may also be incubated overnight.
5. The working stock plate can be stored at –20°C for several months without appreciable product degradation. Avoid repeated freezing and thawing of the original stock plate.
6. Make sure that there are no air bubbles trapped under the coverslip. The slides should be covered with aluminum foil to protect from light during the incubation steps. The incubation time must be uniform for all slides, and 1 h of incubation is recommended. Depending on desired signal to noise, this incubation time may be increased or reduced. Ensure that the solution under the coverslip is evenly distributed.

Acknowledgments

We gratefully acknowledge financial support by MOE (R143-000-394-112), BMRC (R143-000-391-305), CRP (R143-000-218-281), and DSO National Laboratories. We thank R. Yada (University of Guelph, Canada) and M. Wolfe (Harvard) for the kind gifts of HAP (and mutants) and γ-30 cell line, respectively.

References

1. Duffner, J. L., Clemons, P. A. and Koehler, A. N. (2007) A pipeline for ligand discovery using small-molecule microarrays. Curr. Opin. Chem. Biol. 11, 74–82.
2. Uttamchandani, M., Lee, W. L., Wang, J. and Yao, S. Q. (2007) Quantitative inhibitor fingerprinting of metalloproteases using small molecule microarrays. J. Am. Chem. Soc. 129, 13110–13117.
3. Takahashi, M., Nokihara, K. and Mihara, H. (2003) Construction of a Protein-detection system using a loop peptide library

with a fluorescence label. Chem. Biol. 10, 53–60.

4. Uttamchandani, M., Wang, J. and Yao, S. Q. (2006) Protein and small molecule microarrays: powerful tools for high-throughput proteomics. Mol. BioSyst. 2, 58–68.
5. Mastrolorenzo, A., Rusconi, S., Scozzafava, A., Barbaro, G. and Supuran, C. T. (2007) Inhibitors of HIV-1 protease: current state of the art 10 years after their introduction. From antiretroviral drugs to antifungal, antibacterial and antitumor agents based on aspartic protease inhibitors. Curr. Med. Chem. 14, 2734–2748,
6. Chan, E. W. S., Chattopadhaya, S., Panicker, R. C., Huang, X. and Yao, S. Q. (2004) Developing photo-active affinity probes for proteomic profiling-hydroxamate-based probes for metalloproteases. J. Am. Chem. Soc. 126, 14435-14446.
7. Li, Y. M., Xu, M., Lai, M. T., Huang, Q., Castro, J. L., DiMuzio-Mower, J., Harrison, T., Lellis, C., Nadin, A., Neduvelil, J. G., Register, R. B., Sardana, M. K., Shearman, M. S., Smith, A. L., Shi, X. P., Yin, K. C., Shafer, J. A. and Gardell, S. J. (2000) Photoactivated γ-secretase inhibitors directed to the active site covalently label presenilin 1. Nature, 405, 689–694.
8. Takahashi, Y., Konno, Y., Watanabe, N., Miyashita, H., Sasaki, M., Natsugari, H., Kan, T., Fukuyama, T., Tomita, T. and Iwatsubo, T. (2007) Divergent synthesis of multifunctional molecular probes to elucidate the enzyme specificity of dipeptidic γ-secretase inhibitors. ACS Chem. Biol. 2, 408–418.
9. Chun, J., Yin, Y. I., Yang, G., Tarassishin, L. and Li, Y. M. (2004) Stereoselective synthesis of photoreactive peptidomimetric γ-secretase inhibitors. J. Org. Chem. 69, 7344–7347.
10. Fuwa, H., Hiromotoa, K., Takahashia, Y., Yokoshimaa, S., Kan, T., Fukuyamaa, T., Lwatsuboa, T., Tomitaa, T. and Natsugari, H. (2006), Synthesis of biotinylated photoaffinity probes based on arylsulfonamide γ-secretase inhibitors. Bioorg. Med. Chem. Lett. 16, 4184–4189.
11. Shi, H., Liu, K., Xu, A., and Yao, S. Q. (2009) Small molecule microarray-facilitated screening of affinity-based probes (A*f*BPs) for γ-secretases. Chem. Commun. 5030–5032.
12. Wang, J., Uttamchandani, M., Li, J., Hu, M. and Yao, S. Q. (2006) "Click" synthesis of small molecule probes for activity-based fingerprinting of matrix metalloproteases. Chem. Commun. 3783–3785.

Chapter 6

Nanodroplet Chemical Microarrays and Label-Free Assays

Dhaval Gosalia and Scott L. Diamond

Abstract

The microarraying of chemicals or biomolecules on a glass surface allows for dense storage and miniaturized screening experiments and can be deployed in chemical-biology research or drug discovery. Microarraying allows the production of scores of replicate slides. Small molecule libraries are typically stored as 10 mM DMSO stock solutions, whereas libraries of biomolecules are typically stored in high percentages of glycerol. Thus, a method is required to print such libraries on microarrays, and then assay them against biological targets. By printing either small molecule libraries or biomolecule libraries in an aqueous solvent containing glycerol, each adherent nanodroplet remains fixed at a position on the microarray by surface tension without the use of wells, without evaporating, and without the need for chemically linking the compound to the surface. Importantly, glycerol is a high boiling point solvent that is fully miscible with DMSO and water and has the additional property of stabilizing various enzymes. The nanoliter volume of the droplet forms the reaction compartment once additional reagents are metered onto the microarray, either by aerosol spray deposition or by addressable acoustic dispensing. Incubation of the nanodroplet microarray in a high humidity environment controls the final water content of the reaction. This platform has been validated for fluorescent HTS assays of protease and kinases as well as for fluorogenic substrate profiling of proteases. Label-free HTS is also possible by running nanoliter HTS reactions on a MALDI target for mass spectrometry (MS) analysis without the need for desalting of the samples. A method is described for running nanoliter-scale multicomponent homogeneous reactions followed by label-free MALDI MS spectrometry analysis of the reactions.

Key words: Microarray, Protease, Mass spectrometry, Glycerol

1. Introduction

Mass spectrometry (MS) is a label-free detection system that overcomes various limitations of fluorescent labeling. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a soft ionization technique providing molecular weight information of charged analytes in a reaction mixture.

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_6,

Its high sensitivity, speed, and relatively high tolerance for salts and buffers make it an ideal method for profiling small molecule libraries (1–3) and complex biomolecules such as kinases, carbohydrates, and lipids (4, 5). With increasing advances in MS instrumentation and automation, MALDI-TOF MS can meet the demands of chemical HTS and chemical-biology applications. The use of MS requires that HTS protocols, MALDI-target layouts, and sample preparation protocols be consolidated into a unified process.

Recently, microarray-based technologies have been coupled to MS to profile protein-small molecule and enzymatic reactions (4, 6, 7). However, assays performed in well plates or microcentrifuge tubes before arraying onto chips for detection do not exploit the scale down or liquid handling power of microarray printing (4, 6). Thus, a need exists to create localized reaction volumes in an array-based MALDI-target format as well as to create a method of rapidly delivering small volumes of fluid to each reaction. While evaporation effects typically prevent the extreme scale down of well-plate reactions to nanoliter volumes (8), the use of water-miscible, low volatility solvents, such as glycerol, enables nanoliter reaction assembly on microarrays (9, 10).

We have previously developed a novel multicomponent solution phase microarray-based technology to screen for biochemical reactions at a nanoliter scale (9, 10). The need for covalent immobilization of biological entities is overcome by utilizing glycerol-based buffers and thus allows for multicomponent reaction assembly. Reaction activation is achieved through aerosol deposition of the target and substrate. This method achieves minimal evaporation, no cross-contamination, high reproducibility, and minimal sample usage (9). The method is applicable for high-throughput drug screening, protease/substrate profiling (10–12), functional phenotyping of protease mixtures (10, 13), and kinase profiling (14).

We have modified this technology platform to provide label-free detection using MALDI-TOF MS, hence overcoming the limitations of fluorescence-based assay formats. This is one of the first methods to our knowledge to perform on-target multicomponent nanoliter reaction assembly, activation, and detection using a microarray format.

2. Materials

2.1. Consumables

1. 384-well printing plates compatible with microarrayer (Genome Sciences, OmniGrid).
2. Glycerol (HPLC Grade).
3. Adhesive plate sealing foil.

4. 10 mg/ml of sinapinic acid in 50% ethanol.
5. Human thrombin and plasminogen-depleted human fibrinogen (Enzyme Research Laboratories, South Bend, IN).
6. Fibrinopeptide B-Tyr and 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (Sigma, St. Louis, MO).
7. Benzamidine, a thrombin inhibitor (156.61 Da) (Fisher Biotech, Fair Lawns, NJ).
8. All proteases, peptides, and inhibitors were reconstituted in manufacturer recommended buffers.
9. Blank mass spectrometry plates (Applied Biosystems, Foster City, CA) compatible with the Voyager DE-PRO instrument.

2.2. Equipment

1. UMPII flow pump (World Precision Instruments, Saratoga, FL).
2. MALDI Mass Spectrometer (Voyager DE-PRO instrument).
3. OmniGrid Microarrayer (Genome Sciences) or equivalent.
4. Ultrasonic nozzle (Sonotek Corp.).

3. Methods

Utilization of glycerol in the print buffer and the aerosolization process circumvents the need for crosslinking compounds to the surface. DMSO, the solvent of choice for most compound libraries is fully miscible in glycerol, which is a water mimic capable of forming multiple hydrogen bonds. Thus, solubilized compounds from a screening library can be directly printed on to the target plate, eliminating the synthetic steps required for derivatization of the compounds and the plates. This reduces nonspecific binding and enzyme accessibility issues caused by crosslinking to the surface (15, 16), thereby increasing assay efficiency. Additionally, glycerol provides the necessary lubrication for enzymatic catalysis (17, 18) and minimizes compound precipitation, protein aggregation/denaturation, and nonspecific interactions. While glycerol can potentially reduce assay sensitivity by modulating the binding equilibria through hydrophobic or osmolarity effects (19), correlation studies between microarray and well plate data indicate no dramatic variations in protease specificity (9, 10).

The protocol of the assay is summarized as follows:

1. Prepare 384-well printing plates (compounds or biomolecules) in 10–25 µL of 50% glycerol/water (HPLC grade glycerol) (see Note 1). Plates (round bottom) can be sealed with adhesive foil or preferably foil heat sealed for storage either at refrigerated or frozen conditions depending on the stability

of the library in use. Assay-specific MS standards and calibrants can easily be added within each well prior to the printing of microarrays. Exploiting the low volatility of glycerol, distinct reaction spots are contact printed on a stainless steel blank MS plate (Fig. 1a, b). Each spot had an average volume of 1.5 nL with spot diameter of 500 μm and center-to-center spacing of 1,000 μm, thereby allowing up to 9,600 discrete reactions (25×384) on a single target plate.

2. After microarray printing of well plates on MALDI target plates, the reactions are activated by aerosol deposition of

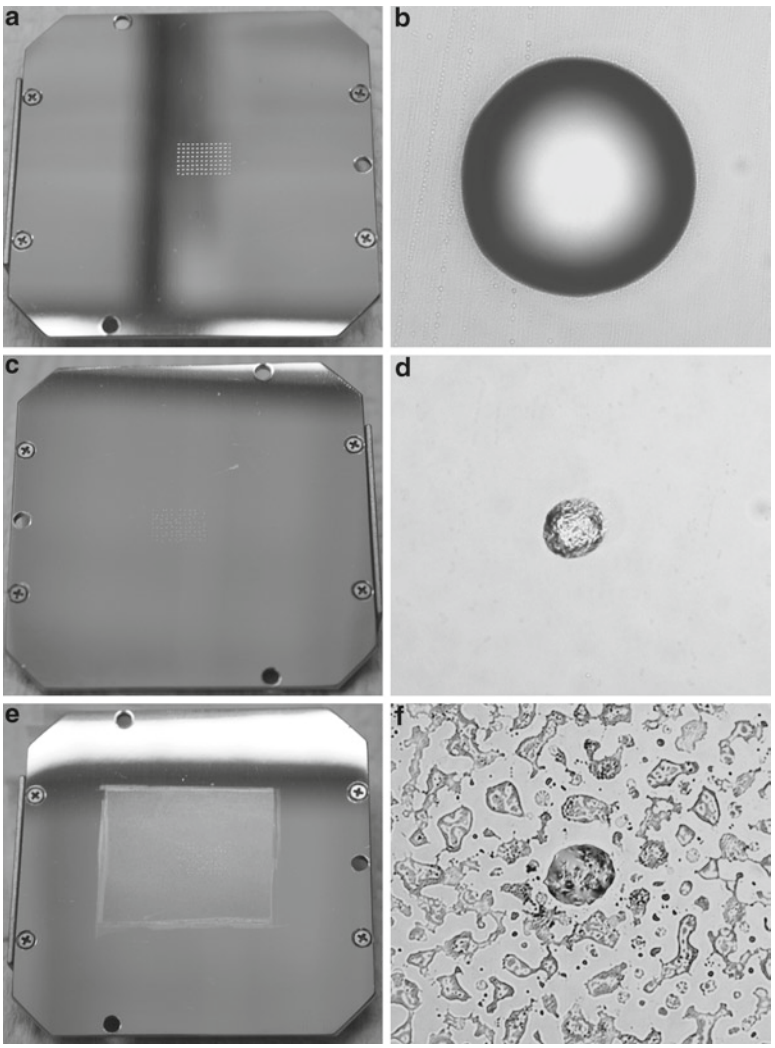


Fig. 1. Label-free detection of pin-printed nanoliter scale reactions conducted on MALDI MS targets. A 8×12 array of reactions were printed using an OmniGrid microarrayer containing varying concentrations of fibrinogen and a fibrinopeptide MS standard. Certain reactions contained 1 mM benzamidine. The array was activated with thrombin and incubated under humidity (**a** and **b**), the glycerol was removed (**c** and **d**), and then spray coated with Maldi matrix (**e** and **f**).

enzyme (see Note 2). Aerosol formation, carrier gas, and target translation during deposition are described below.

3. Incubate targets for 0.5–6 h depending on reaction kinetics at 37°C in a humidity chamber to achieve 95% relative humidity (final water concentration in the reaction is dictated by liquid-vapor equilibrium to achieve ~5% final glycerol by volume in the adherent nanodroplets). Removal of the target plates to room conditions will immediately reduce the water content of the nanodroplets to essentially stop the reaction.
4. Vacuum dry the target plates for 6–24 h. Aerosol deposition of MALDI-matrix (10 mg/ml of sinapinic acid in 50% ethanol) is conducted to form ~10 to 20 μm thick layer of matrix.
5. Targets are analyzed by MALDI-MS (typically 100 laser pulses per sample) using parameters optimized to maximize the detection of assay components.

3.1. Aerosol Generation for Microarray Activation

1. Individual reactants (fibrinogen) were prepared as 50% glycerol/water mixtures in 384-well plates and microarrayed on the metal MALDI targets.
2. Activate reactions by delivering biological samples to the array via aerosol deposition at a liquid flow rate of 400 nL/s using a UMPII flow pump from World Precision Instruments (Saratoga, FL) as previously described (9, 10). Direct a sheath of carrier gas (2.3 L/min air) around the ultrasonic nozzle (Sonotek Corp.) to direct the aerosol to the microarray. The nozzle is placed between 5 and 15 cm above the target, depending on the application. The target is translated underneath the aerosol at a velocity between 2 and 8 cm/min. Sample delivery to the array was achieved by depositing an aerosol generated with an ultrasonic nozzle operating at 120 kHz. Aerosolization of the sample resulted in a fine mist with a median droplet diameter of 18 μm (~3 pL). Aerosol droplets deposited evenly on and around the nonspreading glycerol spots and rapidly evaporated within 7 s without mixing between spots. Accurate and controllable delivery of reactants to each reaction center was achieved by manipulating the aerosolization time and flow rate of the delivered samples. The deposition process was uniform with intraslide spot-to-spot coefficient of variation (CV) of ±16%, interslide CV of <±3%, and overall aerosol deposition efficiency of >95% (9).
3. After evaporation of the deposited mist, a nonvolatile residue of protein is left on the plate surface between the glycerol droplets.
4. After aerosol deposition for reaction activation, incubate the plates at 37°C (95% relative humidity) for up to 4 h.

For imaging purposes, the reactions were performed on glass slides and viewed with a 10× plan lens using a Leica DM–IRBE. Images were acquired using a 12-bit Hamamatsu Peltier-cooled, 1,344 × 1,024 pixel CCD camera.

3.2. Detection Using MALDI-TOF MS

To detect reaction products (analytes) using MALDI-TOF MS, it was necessary to remove glycerol from the reaction mixture. This was achieved by applying a vacuum to the target plate using a SpeedVac (37°C, 6 h). Glycerol removal resulted in the reduction of spot size diameter of 100 μm with a concomitant concentration of analyte crystals. A thin uniform layer of matrix, 10 mg/ml of sinapinic acid in 50% ethanol, was then aerosolized onto the plate to provide a protonation source for the analytes. After evaporation of matrix solvent, the analytes were ready for detection using a Voyager DE-PRO MALDI-TOF MS instrument from Applied Biosystems (Foster City, CA). After reaction activation and incubation, the arrays were prepared for MS analysis. Unlike fast atom bombardment (FAB), which can analyze reactions in solution phase by utilizing liquid matrices such as glycerol (20), MALDI requires a solid phase format and crystalline matrix (21). Glycerol from the arrays was extracted by placing the plates in a chamber subjected to high vacuum. The extraction resulted in a significant reduction in spot diameter ~100 um and left behind evenly distributed crystal deposits of the reaction products (Fig. 1c, d). With the laser beam diameter (oval, ~400 × 600 um – personal communication, Applied Biosystems technical help desk) being greater than the spot diameter, an entire spot could be ablated without movement, reducing sample analysis time.

1. Operate the mass spectrometer in positive reflectron mode with an accelerating voltage of 20 kV. Samples were ablated with a nitrogen laser operated at 337 nM with a laser repetition rate of 20 Hz (3 ns pulse). A delayed extraction time of 150 ns was applied to stabilize the ions produced by laser desorption before entering the time-of-flight analyzer.
2. Fire an average of 50–100 laser pulses for each sample to obtain signal-to-noise ratio statistics for each spectrum (see Note 3).
3. A total of 100 pulses were generally sufficient to ablate the entire matrix-analyte crystal mixture obtained from each 1.5 nL enzyme reaction.
4. Run all samples in an automated mode with the laser intensity optimized (signal-to-noise ratio of >80) to analyze signals in each spot. On average, about 10–15 s was spent per spot sample for data acquisition, sample repositioning, and laser firing.

3.3. Cleavage of Fibrinogen Using Thrombin

1. Aliquote plasminogen-depleted, human fibrinogen was aliquoted at 40 mg/ml in 10 mM phosphate, 20 mM citrate, and 0.15 M NaCl (pH 7.4).
2. Perform further dilutions in 30% glycerol to final concentrations of 100 μg/ml, 10 μg/ml, 1 μg/ml, 100 ng/ml, 10 ng/ml, and 1 ng/ml. Prepare for each sample a negative control comprising fibrinogen spiked with 250 μM benzamidine, a thrombin inhibitor.
3. Add 500 ng/ml (final concentration) of fibrinopeptide B–Tyr (MW: 1715.7) to each sample as an internal calibrant to enable ratiometric quantification studies.
4. Print blank spots to check for cross-contamination. Typically, six replicates were printed for each concentration and negative controls, on the blank plate and activated with 500 nM of human thrombin (50 mM sodium citrate, 0.2 M NaCl, 0.1% PEG, pH 6.5).
5. The reaction is allowed to incubate for 3 h before extracting glycerol from the reaction mixture and spraying the microarray with sinapinic acid. Analyze and detect the reaction products of fibrinogen cleaved with thrombin, fibrinopeptide A (MW: 1536.6) and B (MW: 1569.6) were detected with MALDI-TOF MS. Typical results obtained are shown in Fig. 2. The crystallized arrays were further subjected to an aerosol deposition of sinapinic acid in 50% ethanol resulting in redissolution of the crystals. The thin matrix layer quickly evaporated leaving behind an intimate mixture of analyte-matrix microcrystal structures (Fig. 1e, f). The fine microcrystal deposits and reduced spot size resulted in increased sensitivity and spot-to-spot reproducibility (see Note 4). Benzamidine-spiked spots provided a negative control for each concentration and fibrinopeptide B – Tyr was used as an internal calibrant in each sample spot. Spots lacking fibrinogen and internal calibrant were also arrayed for the detection of cross-contamination between spots. Peaks for the reaction products, fibrinopeptides A and B along with the internal calibrant were easily detected within the 1,000–2,000 m/z range for all the relevant sample spots (Fig. 2a–c). MALDI-TOF analysis on the blank spots was devoid of any peaks within the 1,000–2,000 m/z range showing the absence of any cross contamination. Also, no cleavage products were detected in the sample spots spiked with benzamidine (Fig. 2d). Mass spectras were baseline normalized for quantification of the reaction products. The CV's for each concentration ($n=6$) were <15% showing the spot-to-spot reproducibility and sensitivity making the method amenable to high-throughput screening of small molecule libraries to identify inhibitors.

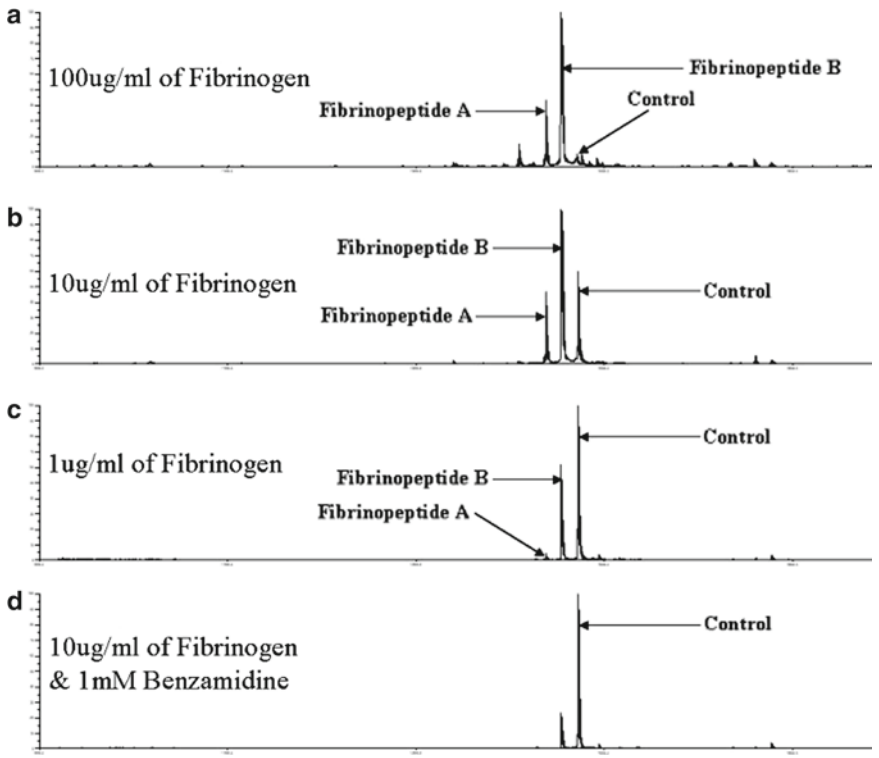


Fig. 2. Individual reactions were then subjected to MALDI for the analysis of fibrinopeptides A and B. A large signal was seen at 1 μg/ml (**a**), 10 μg/ml (**b**), and 100 μg/ml (**c**) of fibrinogen (3, 30, and 300 nM) treated with thrombin, but not in screening reactions containing benzamidine (**d**). Conditions of the MALDI MS were: Voyager- DE-PRO (sinapinic acid matrix, Glu1-Fibrinopeptide B (1 ug/ml) control, reflector mode, positive polarity, 1,000–2,000 Da acquistion range, 20,000 V accelerating voltage, 2,033 laser intensity (20.0 Hz; 100/spectrum), extraction delay time: 150 ns, grid voltage: 75%, Guide wire voltage 0.002%.

4. Notes

1. Glycerol is very viscous and can cause challenges in liquid handling. If necessary, the addition of 5–10% by vol. dry ethanol to glycerol solutions will dramatically reduce the viscosity of the solution for automated liquid handling. This ethanol will evaporate within 15 min after the glycerol is delivered.
2. Given the small feature size of the nanodroplets and the absence of covalent immobilization, initiating thousands of individual microarray-based reactions using standard liquid handling techniques presents a unique challenge. Piezo or ink-jet dispensers have exacting surface tension or viscosity requirements and are prone to clogging (Hertzberg). Bubble entrapment in displacement dispensers can cause spitting and pin tool dispensing requires difficult readdressing of reaction

locations. Either aerosol deposition or acoustic dispensing (22, 23) allows relatively straightforward and robust activation of nanoliter droplet reactions on a microarray. A single aerosol deposition run is capable of delivering a total of 50 μL of liquid sample to five target plates. For enzyme reactions, the cycle time between aerosol deposition of subsequent enzymes and substrates was 250 s to allow for the complete evaporation of the aerosol between the spots.

3. Given the small spot size after glycerol extraction, the laser diameter (~400 × 600 um, oval shaped) usually encompassed the entire spot resulting in high reproducibility, increased the sensitivity and reduced analysis time.
4. Desalting of the sample was not required due to a combination of: (1) small reaction nanoliter-scale reaction volumes, (2) high concentrations of a few reagents typical of HTS reactions, and (3) the redissolution of the crystals with sinapinic acid matrix thereby allowing salt diffusion from the reaction center.

References

1. Tholey, A., Wittmann, C., Kang, M. J., Bungert, D., Hollemeyer, K., and Heinzle, E. (2002) Derivatization of small biomolecules for optimized matrix-assisted laser desorption/ionization mass spectrometry, *J Mass Spectrom 37*, 963–973.
2. Siuzdak, G. (1994) The emergence of mass spectrometry in biochemical research, *Proc Natl Acad Sci U S A 91*, 11290–11297.
3. Glish, G. L., and Vachet, R. W. (2003) The basics of mass spectrometry in the twenty-first century, *Nat Rev Drug Discov 2*, 140–150.
4. Min, D. H., Su, J., and Mrksich, M. (2004) Profiling kinase activities by using a peptide chip and mass spectrometry, *Angew Chem Int Ed 43*, 5973–5977.
5. Harvey, D. J. (1999) Matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates, *Mass Spectrom Rev 18*, 349–450.
6. Min, D. H., Tang, W. J., and Mrksich, M. (2004) Chemical screening by mass spectrometry to identify inhibitors of anthrax lethal factor, *Nat Biotechnol 22*, 717–723.
7. Shen, Z., Go, E. P., Gamez, A., Apon, J. V., Fokin, V., Greig, M., Ventura, M., Crowell, J. E., Blixt, O., Paulson, J. C., Stevens, R. C., Finn, M. G., and Siuzdak, G. (2004) A mass spectrometry plate reader: monitoring enzyme activity and inhibition with a Desorption/Ionization on Silicon (DIOS) platform, *Chembiochem 5*, 921–927.
8. Wolcke, J., and Ullmann, D. (2001) Miniaturized HTS technologies - uHTS, *Drug discov today 6*, 637–646.
9. Gosalia, D. N., and Diamond, S. L. (2003) Printing chemical libraries on microarrays for fluid phase nanoliter reactions, *Proc Natl Acad Sci U S A 100*, 8721–8726.
10. Gosalia, D. N., Salisbury, C. M., Ellman, J. A., and Diamond, S. L. (2005) High throughput substrate specificity profiling of serine and cysteine proteases using solution-phase fluorogenic peptide microarrays, *Mol Cell Proteomics 4*, 626–636.
11. Ma, H., Horiuchi, K. Y., Wang, Y., Kucharewicz, S. A., and Diamond, S. L. (2005) Nanoliter homogenous ultra-high throughput screening microarray for lead discoveries and IC50 profiling, *Assay drug dev technol 3*, 177–187.
12. Simmons, G., Gosalia, D. N., Rennekamp, A. J., Reeves, J. D., Diamond, S. L., and Bates, P. (2005) Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry, *Proc Natl Acad Sci U S A 102*, 11876–11881.
13. Gosalia, D. N., Salisbury, C. M., Maly, D. J., Ellman, J. A., and Diamond, S. L. (2005) Profiling serine protease substrate specificity with solution phase fluorogenic peptide microarrays, *Proteomics 5*, 1292–1298.
14. Horiuchi, K. Y., Wang, Y., Diamond, S. L., and Ma, H. (2006) Microarrays for the functional analysis of the chemical-kinase interactome, *J Biomol Screen 11*, 48–56.

15. Johnson, C. P., Jensen, I. E., Prakasam, A., Vijayendran, R., Leckband, D., and 2003. (2003) Engineered protein a for the orientational control of immobilized proteins, *Bioconjug chem 14*, 974–978.
16. Vijayendran, R. A., and Leckband, D. E. (2001) A quantitative assessment of heterogeneity for surface-immobilized proteins, *Anal Chem 73*, 471–480.
17. Klibanov, A. M. (1997) Why are enzymes less active in organic solvents than in water?, *Trends biotechnol 15*, 97–101.
18. Zaks, A., and Klibanov, A. M. (1988) The effect of water on enzyme action in organic media, *J Biol Chem 263*, 8017–8021.
19. Xavier, K. A., Shick, K. A., Smith-Gil, S. J., and Willson, R. C. (1997) Involvement of water molecules in the association of monoclonal antibody HyHEL-5 with bobwhite quail lysozyme, *Biophys J 73*, 2116–2125.
20. Barber, M., Bordoli, R. S., Sedgwick, R. D., and Tyler, A. N. (1982) Fast atom bombardment mass spectrometry of the angiotensin peptides, *Biomed Mass Spectrom 9*, 208–214.
21. Beavis, R. C., and Chait, B. T. (1989) Cinnamic acid derivatives as matrices for ultraviolet laser desorption mass spectrometry of proteins, *Rapid Commun Mass Spectrom 3*, 432–435.
22. Wong, E. Y., and Diamond, S. L. (2008) Enzyme microarrays assembled by acoustic dispensing technology, *Anal biochem 381*, 101–106.
23. Wong, E. Y., and Diamond, S. L. (2009) Advancing microarray assembly with acoustic dispensing technology, *Anal Chem 81*, 509–514.

Chapter 7

Nanodroplet Microarrays for High-Throughput Enzyme Screening

Kang L. D. Aw, Shao Q. Yao, and Mahesh Uttamchandani

Abstract

We describe here a method for the continuous assessment of enzymatic activity using microarrays. By uniformly coating fluorogenic substrates on slides, we generated surfaces capable of detecting enzymatic activity. The enzymes were deposited on the arrays in segregated droplets using standard microarrayers. Surfaces were developed for assessing the activities of both proteases and phosphatases, hence capitalizing on microarray technology to perform miniaturized high-throughput screens for these, as well as potentially any other, classes of enzyme. This offers an unprecedented ability for performing solution-phase enzymatic assays in *nanoliter* volumes on microarrays, in contrast to *microliter* volumes typically required in microplate-based assays, thereby reducing the amounts of reagent(s) required by anywhere from a hundred to a thousand-fold. This new approach thus provides a potentially more cost-effective, label-free enzyme screening technique. A single slide is able to accommodate several thousand assays, facilitating the assessment of both dose and time-dependent inhibition parameters in a single run.

Key words: Small molecule microarrays, High-throughput screening, Metalloproteases, Hydroxamate peptides, Enzyme assays, Chemical libraries, Inhibitor fingerprinting

1. Introduction

Enzymes are responsible for catalyzing all biological pathways, making them an indispensible group of proteins that support life in all its forms. Disruptions to enzymes and their functions can detrimentally affect cellular functions and metabolic exchanges, thereby causing a variety of diseases ranging from cancer and arthritis to Alzheimer's disease and cardiovascular disorders. A better handle over diseases may thus be achieved through a deeper understanding of the roles of enzymes and the processes they control. Platforms, such as the microarray, can facilitate the

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_7,

characterization and annotation of enzyme activity. We have developed a microarray workflow and methodology that enables not only the characterization of enzyme activity using microarrays, but also the screening of inhibitors in a high-throughput manner. This may be further applied to rapidly screen agonists or antagonists against target enzymes for the discovery of therapeutic leads.

Of the many different types of enzymes, proteases, kinases, and phosphatases are among the largest group of proteins and perhaps the most important (1). They have been involved in numerous physiological processes such as cell differentiation, signal transduction, host defense, and apoptosis (2). Minute alterations in expression and regulation of these enzymes can lead to debilitating effects. We have developed microarray surfaces that facilitate the screening of broad classes of enzymes, namely phosphatases and proteases. These arrays may be applied in two separate contexts. First is the ability to carry out functional annotation and discovery of enzymes (3). Second is the high-throughput screening of enzyme agonist or antagonists in an activity-dependent manner for lead discovery or protein fingerprinting (4). Both these approaches are illustrated here using metalloproteases as models. The method is, however, generic to many other classes of enzymes, as long as the corresponding fluorogenic enzyme substrate is available for application.

Our overall approach is illustrated in Fig. 1. Using this approach, one is able to carry out activity-based characterization as well as inhibitor screens of multiple enzymes simultaneously on a single glass slide. Key to our strategy is the application of enzymes by robotic spotting onto surfaces coated with fluorogenic substrates. This microarrayer dispenses subnanoliter volumes accurately and uniformly at predefined locations across the glass slide, thereby creating individual microreactors. This facilitates the variation of different parameters such as buffer conditions, pH, across a series of enzymes for simultaneous comparison and testing. A traditional microplate will require about 100 μL of flourogenic substrate (in this case, Bodipy Casein) for a hundred arrays of reaction wells, whereas just 6 μL of flourogenic substrate is sufficient for 6,148 separate microassays conducted on a slide surface surface of 880 mm^2 (250 μm spot diameter and a spot spacing of 500 μm), thereby enhancing throughput and reducing screening costs. Additionally, the use of enzymes in our approach requires no physical immobilization of the enzymes, thereby overcoming the usual limitation of microarrays where enzymes or inhibitors have to be immobilized or labeled resulting in a loss of activity or unfavorable orientations.

Several groups have also explored similar strategies (5). Salisbury et al. have used peptide derivatives containing coumarin as fluorogenic substrates to screen against different hydrolytic enzymes on microarrays (6). The selective hydrolysis of flourogenic

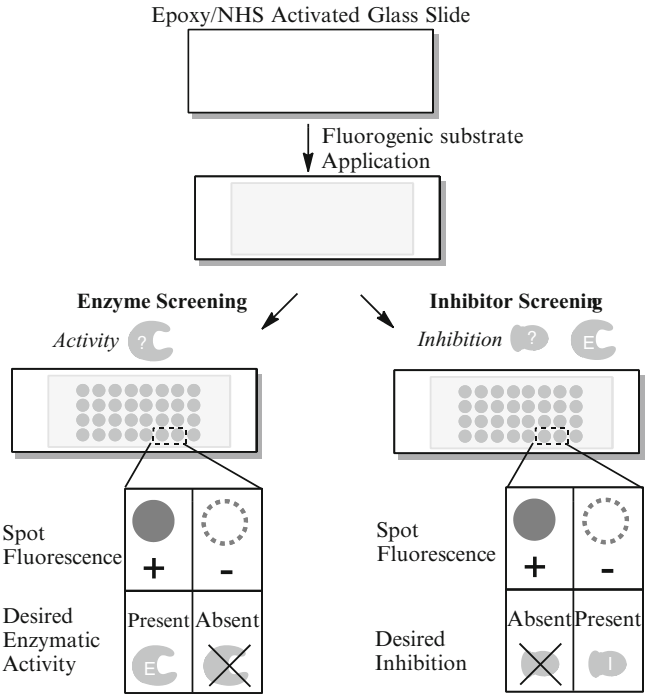


Fig. 1. A strategy for rapid screening of enzymes using microarrays. Fluorogenic substrates were first used to coat glass slides. These coated slides may then be applied either to screen proteins for annotation of their function, or for the discovery of inhibitors against target enzymes of interest.

coumarins through enzymatic activity produced patterns of fluorescence, thus revealing the enzyme's substrate preference. Gosalia and Diamond also printed small molecules in glycerol droplets on a microarray, followed by enzyme application via aerosol. This was applied for inhibitor screening of caspases and serine proteases (7, 8). These strategies only enable, however, one enzyme to be studied on any given slide. Our strategy, in comparison, offers the versatility for both enzymes and inhibitors to be analyzed simultaneously on the same slide. It thus provides an attractive solution for high-throughput enzyme screening.

2. Materials

2.1. Fabrication of Epoxy Slides

1. Sulfuric acid.
2. 30% hydrogen peroxide (Kanto Chemicals).
3. 1% 3-glycidopropyltrimethoxysilane (Sigma-aldrich, cat. No. 440167).
4. Acetic acid.
5. Ethanol.

6. MilliQ H_2O (18.2 Ω, 4 ppb).
7. Nitrogen gas.

2.2. Bodipy Casein Slides Derivatization

1. 1.0 mg/mL Bodipy Casein Working solution: 200 µL of 0.1 M sodium carbonate, pH 8.3 is added to the lyophilized substrate of EnzChek Protease Assay Kit (Invitrogen). Either Tr-X bodipy casein or bodipy casein may be used, as long as the scanner is equipped with the appropriate filter sets, 589/617 nm and 490/528 nm, respectively (see Note 1).
2. Quenching solution: 0.5 mM glycine solution in phosphate buffered solution (PBS) pH 7.4.

2.3. Printing Slides

1. 10–0.01 mg/mL of enzymes prepared in 50 mM Tris-HCl buffer (pH 8.0, 5% glycerol).

2.4. Synthesis of Small molecule Library on Solid Support

1. Rink amide-AM resin (GL Biochem).
2. Hydrochloric acid.
3. 20 proteinogenic Fmoc protected amino acids (GL Biochem).
4. Fmoc-Lys(Biotin)-OH (GL Biochem).
5. O-Benzotriazole- *N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU; GL Biochem).
6. O-(7-Azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) (GL Biochem).
7. N-Hydroxybenzotriazole (HOBt) (GL Biochem).
8. 2,4,6-Collidine (Sigma-Aldrich).
9. Piperidine (Acros Organics).
10. Trifluoroacetic acid (TFA) (Sigma-Aldrich).
11. Triisopropylsilane (TIS) (Sigma-Aldrich).
12. *N*,*N*-Diisipropylethylamine (DIEA) (Sigma).
13. Solvents: Acetone, Acetonitrile, Dichloromethane, Diethyl ether, Dimethylformamide (DMF), Methanol (Tedia).

2.5. Equipment

1. Clean Glass Slides (22 × 60 mm) (Fisher Scientific, Pittsburgh, PA).
2. Microscope Cover Slips (22 × 40 × 0.15 mm) (Matsunami Glass, Japan).
3. Metal slide racks and glass slide staining dish (Electron Microscopy Sciences).
4. Polypropylene slide staining dish and rack (Kartell).
5. Stealth Micro Spotting Pins (TeleChem International, Ca).
6. OmniGrid Microarrayer (Harvard Bioscience, Massachusetts).

7. GenePix 4000B Scanner (MDS Analytical Technologies, Ontario).
8. LC-MS workstation.

2.6. Software

1. OmniGrid Gridder Software (or equivalent).
2. Gene Pix Pro Microarray Image Analysis Software (or equivalent).
3. Microsoft Excel (or equivalent).

3. Methods

The strategy works by coating glass slides surfaces homogenously with commercially available fluorogenic sensors that target proteases. The substrate used employ intramolecularly quenched casein derivatives, which upon hydrolysis, releases a strong fluorescence readout. The fluorescence intensity is proportional to the activity of the protease, thus allowing activity-based measurements to be performed. Typical results obtained are showcased in Figs. 2 and 3.

3.1. Fabrication of Epoxy Slides

Glass slides are cleansed and soaked in piranha solution (70% sulfuric acid: 30% hydrogen peroxide) to remove any organic contaminants and oxidize the silane surface (see Note 2).

1. Use the polypropylene staining dish to contain the piranha solution. The slides may remain soaked in this solution till ready to use (see Note 3).
2. The slides are thereafter transferred to a stainless steel rack and washed copiously with distilled water and dried.
3. Prepare a 400 mL solution containing 1% 3-glycidopropyltrimethoxysilane in 95% ethanol containing 16 mM acetic acid (sufficient for one rack of 30 slides).
4. Premix the solutions in a glass jar staining jar for 10 min and add in the slide in the metal rack. Continuously stir the solution using a magnetic stirrer.
5. Incubate the slides for 1–2 h.
6. Remove the slides from the solution and rinse copiously with ethanol. Air-dry the slides to remove any residual ethanol.
7. Transfer the slide tray to a deep well dish and cure at 150°C for at least 2 h.
8. Subsequently, cool the slides to room temperature and rinse them with ethanol. Air-dry the slides or to speed up the drying

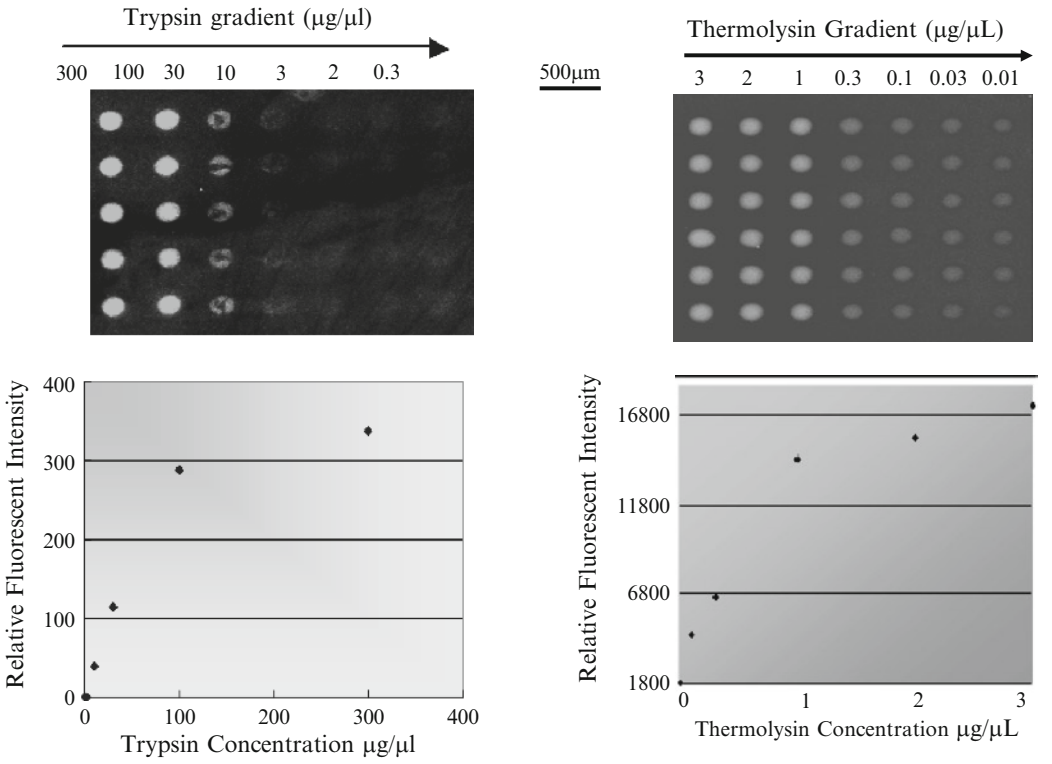


Fig. 2. Dilution series of trypsin and thermolysin respectively printed on bodipy casein coated slides. The intensity profiles are represented by a graphical plot (bottom). (Reprinted from ref 3 with permission from Elsevier).

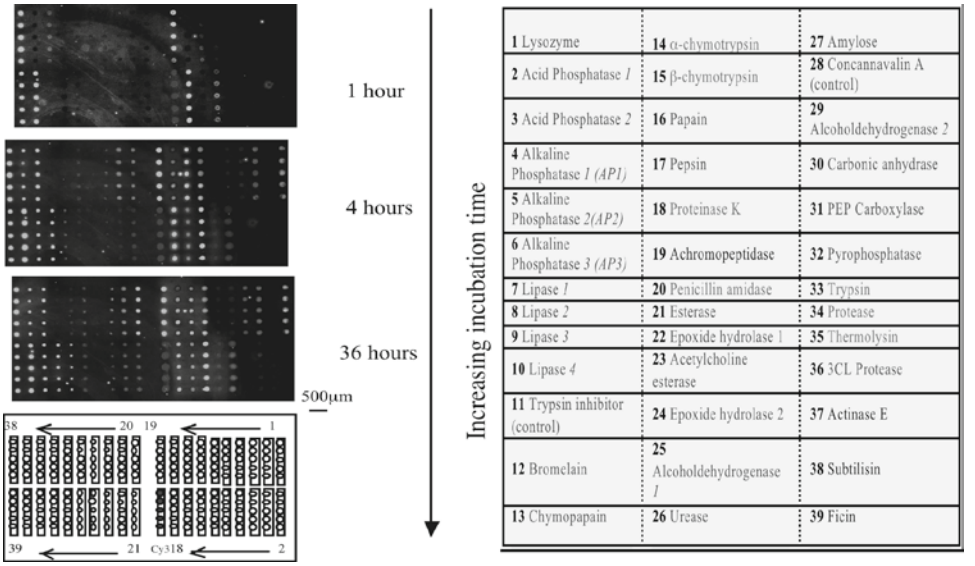


Fig. 3. Microarray images taken at different time points for a panel of enzymes on bodipy casein slides. It can be seen that positive measurements of proteolytic activities were obtained in a time-dependent manner. The controls or nonproteolytic enzymes did not display significant fluorescent readouts. (Reprinted from ref 3 with permission from Elsevier).

process, use centrifugation or a stream of dry nitrogen/compressed air to dry the slides.

9. Slides may be stored at room temperature until ready for use.

3.2. Preparing Bodipy Casein Slides

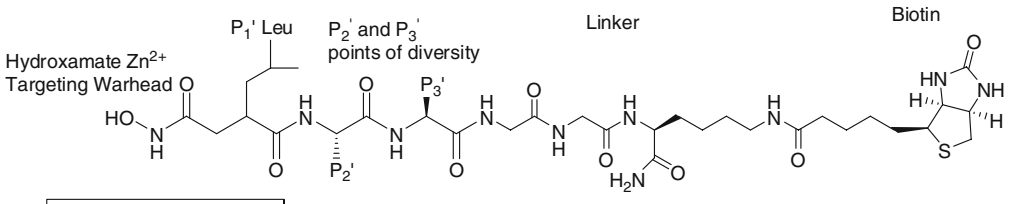
1. Dilute 6 μL of the original working stock solution of bodipy casein in 44 μL of PBS.
2. Apply reaction mix on epoxide-functionalized slides using cover slip method (see Note 4)
3. Incubate slides for 2 h in an enclosed light protected humid chamber to prevent drying up of the reaction mix.
4. After 2 h of incubation, quenched slides are quenched in 0.5 mM glycine in 1× PBS buffer.
5. Rinse in MilliQ water and dry. Store at 4°C, in the dark.

3.3. Printing Enzymes on Microarrays to Detect Enzymatic Activity

1. Prepare the enzymes at desired concentrations (usually between 10 and 0.01 mg/mL) in 50 mM Tris-HCl buffer (pH 8.0, 15% glycerol) (see Note 5).
2. The enzymes are then dispensed into 384 wells plates using multichannel pipettes.
3. Shake the 384 wells plate to mix and spin down. The plate is kept on ice until printing.
4. Design grids using the gridder software to plan the arrangement of the spots on the slide such that the enzymes are spotted at least twice on the slide. Blotting may be incorporated into the printing schedule to improve spot quality across the slide.
5. Rinse to rehydrate the coated slides and dry prior to loading the slides on the spotter. Ensure the TR-X casein coated surface is facing upward.
6. Spot the enzymes using an OmniGrid Microarrayer with a spot spacing of 500 μm using a SMP8B pin, creating spots of diameter of approximately 350 μm. (see Notes 6 and 7)
7. Slides are printed in a humid environment with saturation of 85% to minimize evaporation of the droplets and in the absence of light.

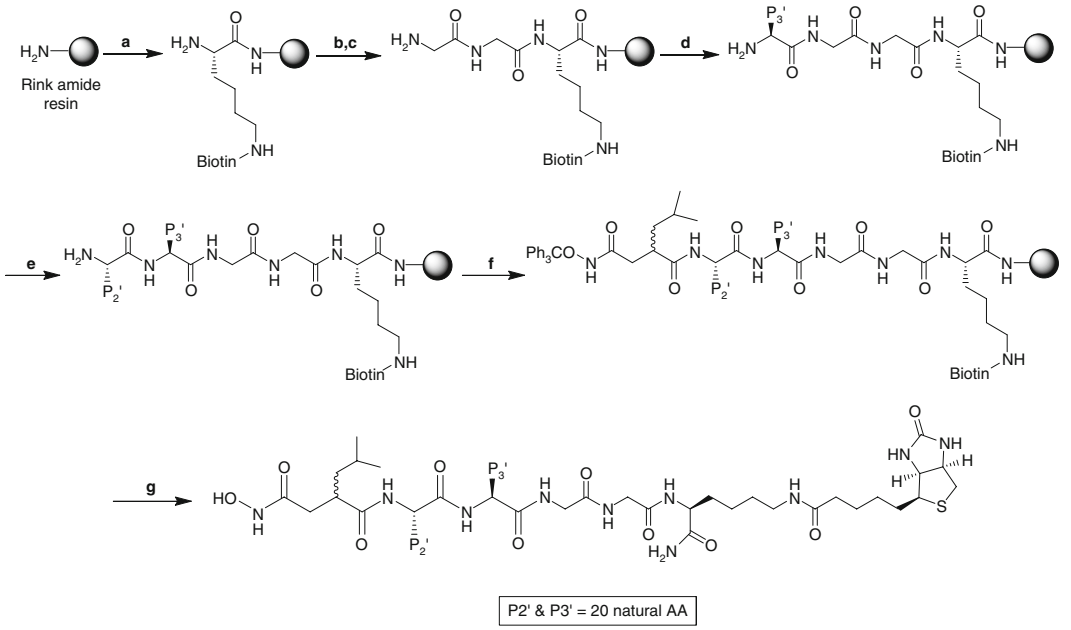
3.4. Design and Synthesis of Inhibitor Library

The design of the small molecule library is shown in Fig. 4 that targets metalloproteases. The synthetic scheme is provided in Scheme 1. The synthesis of the panel of 14 different trityl-protected hydroxamate warheads have been described previously (9). Here, the protocol is described for the synthesis of a 400 member library, using Fmoc solid phase peptide synthesis (10). The procedure may be modified as desired to create various points of



P_1'	P_2'	P_3'	Library Size
L	20 natural amino acids	20 natural amino acids	400 members
1 P_1' warhead			Total: 400 compounds

Fig. 4. Design of small molecule inhibitor library. A 400 member hydroxamate peptide library was designed to target metalloproteases.



Scheme 1. Synthesis scheme of small molecule library. (**a**) (1) Fmoc-Lys(Biotin)-OH, HOBT, HBTU, DIEA, DMF; (2) 20% piperidine/DMF; (**b**) (1) Fmoc-Gly-OH, HOBT, HBTU, DIEA; (2) 20% piperidine/DMF; (**c**) (1) Fmoc-Gly-OH, HOBT, HBTU, DIEA; (2) 20% piperidine/DMF; (**d**) (1) Fmoc-AA(P_3)-COOH, HOBT, HBTU, DIEA; (2) 20% piperidine/ DMF; (**e**) (1) Fmoc-AA(P_2)-COOH, HOBT, HBTU, DIEA; (2) 20% piperidine/DMF; (**f**) (1) 4, CPh_3ONH-Leu-COOH, HATU, 2,4,6-collidine; (2) 20% piperidine/DMF; (**g**) 95% TFA/5% TIS, 2 h.

diversity. It is also manageable for one person to handle this protocol with 400–500 MicroKans independently, which would consume a period of between 8 and 12 days.

1. Calculate the amount of rink amide resin required for library synthesis. The capacity for each MicroKan is 30 mg of resin. This would work out to a total of 12 g of resin for a

400 member library. The theoretical yield of each library member at this scale of synthesis would be 15 μmoles, when using rink amide resin with 0.5 mmol/g resin loading capacity. This quantity is more than sufficient for printing several thousand microarray slides.

2. Weigh 12 g of rink amide resin into a fritted funnel, with a screw cap. Add 35 ml of DMF, cap both ends of the funnel and shake for 2 h to allow the resin to swell. Drain the DMF using suction.
3. Repeat the wash step with 35 ml DMF for a further three times, for 15 min each time with shaking.
4. Deprotect the resin using 20% v/v piperidine in DMF for 1 h with shaking. Drain the piperidine solution using suction.
5. Wash the resin with DMF (35 ml) for three times, 15 min per wash with shaking.
6. Wash with DCM (35 ml) for a further three times for 15 min per wash with shaking.
7. Wash with DMF (35 ml) for a further three times for 15 min per wash with shaking.
8. Transfer the resin from the funnel into a 250 ml glass bottle. Rinse with 25 ml of DMF to ensure all resin is completely transferred. Repeat with another 10 ml of DMF.
9. Couple resin with Fmoc-Lys(biotin)-OH. Weigh out four equivalents (24 mmol) of Fmoc-Lys(biotin)-OH, HBTU, and HOBt in a separate bottle. Dissolve in 80 ml of DMF and add 8 equivalents of DIEA (48 mmol). Mix well, and allow for preactivation by leaving the solution to stand for 15 min.
10. Add the preactivated solution to the resin. Seal the bottle with parafilm and shake overnight (~12 h).
11. Filter the resin using suction through a 350 ml fritted funnel. Ensure that all resin is transferred using small volume rinses with DMF.
12. Wash the resin using 3× DMF, 3× DCM, and 3× DMF, as described in steps 5–7, using 100 ml of solvent per wash.
13. Isolate around ten beads, and perform the ninhydrin test to ensure that the coupling is successful and complete (see Note 8).
14. Transfer the resin to a clean bottle and deprotect Fmoc using 150 ml of 20% piperidine in DMF.
15. Wash as detailed in step 12.
16. Repeat steps 8–15 using Fmoc-Gly-OH in place of Fmoc-Lys(biotin)-OH to couple a glycine residue.
17. Repeat step 16 to add on another glycine residue.

18. Dry the resin under vacuum, using an oil pump for 6 h.
19. Distribute ~30 mg of resin in each of 400 MicroKans reactors. Include an IRORI radiofrequency tag to each reactor and ensure that the cap is fitted on tightly. Load four extra reactors for ninhydrin tests to monitor coupling efficiency.
20. Program the Accutag Synthesis Manager software for a 20 aa × 20 aa = 400-member library.
21. Scan the 400 reactors to encode each tag and sort into twenty 100 ml bottles. Reactors in each bottle will be coupled with the same amino acid.
22. To couple the P_3' residues, prepare preactivated solutions for each of the 20 Fmoc-protected proteinogenic amino acids, using the four equivalent molar excess of amino acid, HBTU, HOBt, and eight equivalent molar excess of DIEA. Prepare these solutions in 50 ml of DMF.
23. Add the twenty different amino acid preactivated solutions to the 20 bottles containing the respective MicroKans. Ensure that all bottles are appropriately labeled.
24. Shake bottles overnight (~12 h).
25. Drain the solutions in each bottle and wash twice (for 15 min) with 60 ml of DMF.
26. Pool all reactors into a 1 L bottle and rinse using 3× DMF, 3× DCM and 3× DMF, as described in steps 5–7, using 500 ml of solvent per wash.
27. Perform Fmoc deprotection using 400 ml of 20% piperidine in DMF.
28. Wash as described in step 26.
29. Repeat steps 21–28 to sort the MicroKans into 20 bottles couple the P_2' position.
30. For the final coupling of the P_1' position, consolidate all MicroKans into a clean 1 L bottle. Proceed with steps 22–28, except use four equivalents of a trityl-protected hydroxamate warhead together with HATU and 2,4,6-collidine (in a 1:1:1.9 ratio) in a 100 ml DMF volume for the coupling step.
31. Perform a final wash with 500 ml of methanol for three times, with shaking each time for 15 min.
32. Dry the resin under vacuum, using an oil pump for 6 h.
33. Prepare the cleavage solution comprising TFA and TIS in the ratio of 19:1. Sort the 400 reactors into individually identified 15 ml tubes. Dispense 1.5 ml of the cleavage solution to each tube, and shake for 3 h. TFA is corrosive and generates fumes, and so perform this step carefully with proper protection in a fumehood.

34. Organize five 96-well deep well plates such that the identities of the samples will be preserved upon transferring all the solutions from each of the 400 15 ml tubes to the plates. Carefully transfer solutions from tubes to plate.
35. Concentrate and remove TFA and TIS using a vacuum evaporator, at a temperature of 35°C at a spin force of 300×*g*, until about 0.1 ml of the solution remains in each well.
36. Add 1.5 ml of cold ether to each well to precipitate the small molecule. Seal the plates with adhesive film and place in a −20°C freezer overnight.
37. Spin down the precipitated products at 1,000×*g* for 30 min, and decant the ether.
38. Dissolve the products in 0.5 ml of DMF. This would give stock concentrations in the range of 1–10 mM. Plates may be sealed and stored for the long term at −80°C.
39. Perform analysis using LC-MS to determine quality and purity of desired products.

3.5. Testing Enzyme Inhibition Using Nanodroplet Microarrays

1. Prepare the enzymes to a 0.5 mg/mL solution in 50 mM Tris-HCl buffer (pH 8.0, 15% glycerol) and mixed with approximately 2 μM of inhibitor.
2. The enzymes and inhibitors are then pipetted into 384 wells plates using multichannel pipettes.
3. Shake the 384 wells plate to mix and spin down. The plate is kept on ice until printing.
4. Design grids using the gridder software to plan the arrangement of the spots on the slide such that the enzymes are spotted at least twice on the slide. Blotting may be incorporated into the printing schedule to improve reproducibility within the slide.
5. Load the required pins and slides in the spotter and make sure the TR-X casein coated side is facing upward.
6. Spot the enzymes using an OmniGrid Microarrayer with a spot spacing of 500 μm using an SMP8B pin, creating spots of diameter of approximately 350 μm. (see Notes 7 and 8).
7. Slides are printed in a humid environment with saturation of 85% to minimize the evaporation of the droplets and if possible in the dark.

3.6. Scanning of the Slides

1. Scan slides on a microarray scanner equipped with the relevant filters. Depending on the nature of the experiment, slides may be scanned periodically, or after a fixed duration of incubation.
2. An example of the typical results obtained is displayed in Figs. 2 and 3 (concentration and time dependent, respectively).

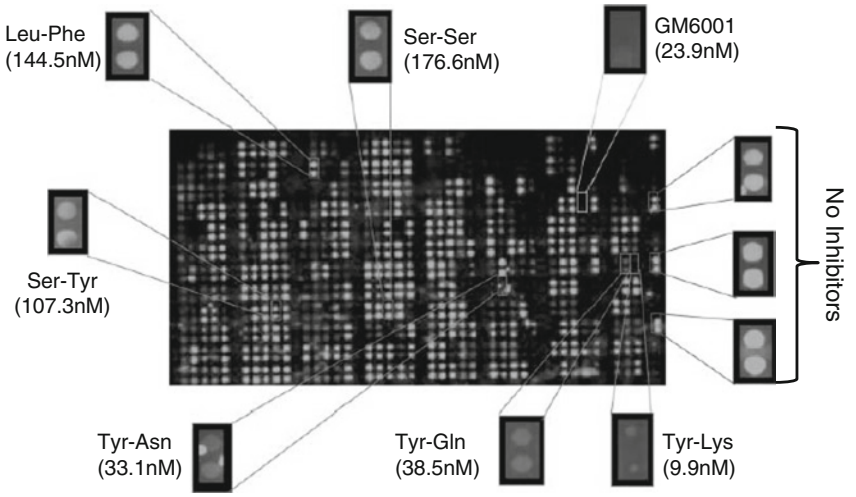


Fig. 5. Nanodroplet microarray results of the 400-member library screened against thermolysin. Samples were spotted in duplicate. Boxes magnify spots of selected inhibitors (labeled by their P_2'–P_3' sequence) with corresponding IC_{50} values in parenthesis. (Reprinted from ref 4 with permission from the Royal Society of Chemistry).

Inhibitor end-point screening results are shown in Fig. 5, with several inhibitors of varying potency displayed inset.

4. Notes

1. Reconstituted fluorogenic substrates can be stored for 2–4 weeks at 4°C. If longer storage is required, fluorogenic substrates should be kept at –20°C. Repeated freezing and thawing should also be avoided to preserve the viability of the substrate.
2. Care must be taken when handling slides to be used in microarray experiments to ensure that at all stages of derivatization, spotting and sample application no dust or dirt come into contact with the planar surfaces. Such particles may cause extraneous fluorescence or result in scratches that could affect the fluorescent readout when the slides are scanned. Ensure that all surfaces and slide racks are clean and rinse these surfaces with ethanol before placing in direct contact with the slides. Gloves, if used, should be of the powder-free variety to ensure that the slides remain uncontaminated even after handling.
3. Piranha solution is highly corrosive. Make sure that proper personal precautions are maintained when handling this solution. Add the hydrogen peroxide to the acid slowly, if not the solution can get very hot very quickly. Solution may be recycled, but is hygroscopic and can absorb moisture from the air

and become diluted over prolonged use. When this happens, prepare fresh solution. Most preparations may be used over 1–2 months.

4. For a 22×40 mm cover slip, a 50 μL reagent is adequate to allow for a uniform coating. Two methods can be used to apply the reagent to the slides. One can either apply the reaction mix to the glass slide followed by the cover slip over the reaction mix or one can apply the reaction mix to the cover slip and then followed by the glass slide onto the reaction mix. Although both methods work similarly and well enough, there are slight differences in the handling and maneuvering of the liquid droplet upon contact of both the slide and cover slip. Either method is fine as long as one is able to produce a uniform distribution of the reaction mix over the cover slip and glass slide without the introduction of air bubbles between the two surfaces.
5. It is preferable to prepare enzymes solution fresh, prior to use. Long-term storage or repeated thawing and freezing may degrade enzyme activity.
6. Spotting volume can be varied by using different pin head sizes. SMP3 stealth pins are very frequently used in microarray fabrication and generally produce spots approximately 100 μm in diameter. As a result, SMP3 pins are more economical in terms of reaction volume, and in addition allowing more nanodroplets to be printed on a single slide. Although this characteristic improves the cost-effectiveness of our proposed methodology, but we found out that when spotting viscous solution, such as enzymes prepared in glycerol, it is more advantageous to use larger pins, such as SMP8 (pins size we used in this proposed methodology), or even SMP15 stealth pins that produces spots in the range of 250–500 μm, respectively. Using SMP8 and SMP 15 pins, visualization and analysis of the spots on the scanner is generally easier. Furthermore, the use of SMP8 pins (pins size used in this methodology) displays a more desirable standard deviation in the reproducibility of the spots as compared to using SMP3, as shown in Fig. 6.
7. During the printing process, the pins are rinsed between samples using two cycles of wash (for 10 s) and sonication (for 20 s) in reservoirs containing 70% ethanol followed by drying under reduced pressure (for 20 s), causing a one minute interval in between samples printing. This interval can be easily changed using the gridder software of the machine, allowing the user to determine the interval required and thus carrying out time and concentration dependent slides printing simultaneously on a single glass slide. As such, the arrayer

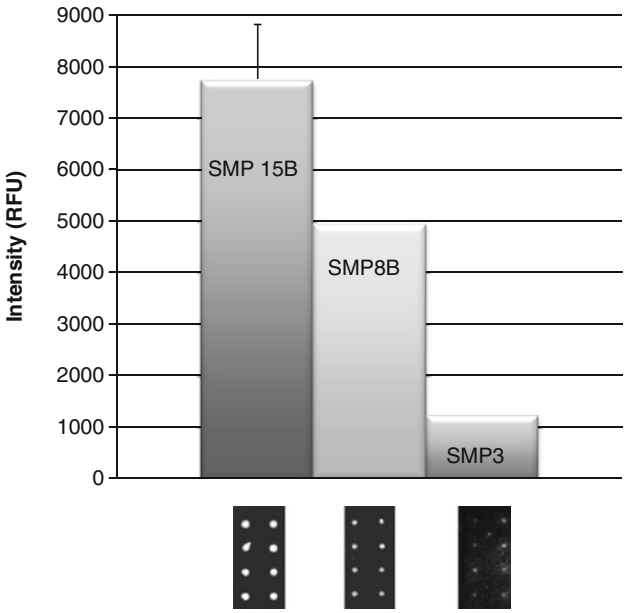


Fig. 6. Thermolysin spots at 1.0 mg/mL on a TR-X bodipy coated Slide using SMP15B, SMP8B, SMP3 pins, and the average plotted values of each corresponding spots. The error bars in the graphs correspond to the standard deviation across the group.

is able to print rows of samples with a fixed time interval in between. This allows the full kinetic read of the samples in a single slide scan.

8. The presence of a blue coloration on the resin and/or solution implies the presence of free amines, and hence indicating incomplete coupling. On the other hand, a straw yellow colour indicates no free amines, and complete coupling. Repeat coupling, if necessary, until a straw yellow colour is obtained. Alternatively, if coupling remains incomplete after several tries, capping may be performed using acetic anhydride.

Acknowledgments

This work is supported by the National University of Singapore (NUS), the Agency of Science Technology and Research (A*Star) of Singapore and the DSO National Laboratories.

References

1. Uttamchandani, M., Lu, C. H., and Yao, S. Q. (2009) Next generation chemical proteomic tools for rapid enzyme profiling, *Acc Chem Res 42*, 1183–1192.
2. Evans, M. J., and Cravatt, B. F. (2006) Mechanism-based profiling of enzyme families, *Chem Rev 106*, 3279–3301.
3. Uttamchandani, M., Huang, X., Chen, G. Y., and Yao, S. Q. (2005) Nanodroplet profiling of enzymatic activities in a microarray, *Bioorg Med Chem Lett 15*, 2135–2139.
4. Wang, J., Uttamchandani, M., Sun, L. P., and Yao, S. Q. (2006) Activity-based high-throughput profiling of metalloprotease inhibitors using small molecule microarrays, *Chem Commun (Camb)*, 717–719.
5. Babiak, P., and Reymond, J. L. (2005) A high-throughput, low-volume enzyme assay on solid support, *Anal Chem 77*, 373–377.
6. Salisbury, C. M., Maly, D. J., and Ellman, J. A. (2002) Peptide microarrays for the determination of protease substrate specificity, *J Am Chem Soc 124*, 14868–14870.
7. Gosalia, D. N., Salisbury, C. M., Ellman, J. A., and Diamond, S. L. (2005) High throughput substrate specificity profiling of serine and cysteine proteases using solution-phase fluorogenic peptide microarrays, *Mol Cell Proteomics 4*, 626–636.
8. Gosalia, D. N., and Diamond, S. L. (2003) Printing chemical libraries on microarrays for fluid phase nanoliter reactions, *Proc Natl Acad Sci U S A 100*, 8721–8726.
9. Uttamchandani, M., Wang, J., Li, J., Hu, M., Sun, H., Chen, K. Y., Liu, K., and Yao, S. Q. (2007) Inhibitor fingerprinting of matrix metalloproteases using a combinatorial peptide hydroxamate library, *J Am Chem Soc 129*, 7848–7858.
10. Chan, W. C., and White, P. D. (2000) *Fmoc solid phase peptide synthesis: a practical approach*, Oxford University Press, New York.

Chapter 8

The Application of the Chemical Array for Biological Study

Isao Miyazaki, Siro Simizu, and Hiroyuki Osada

Abstract

The identification of specific interactions between small molecules and human proteins of interest is a fundamental step in chemical biology and drug development. The small molecules that bind to specific proteins can be used as tools to study the functions of proteins and biological processes in cells. We have developed an efficient method to obtain novel binding ligands of human proteins by a chemical array approach. Our method includes the use of cell lysates that express proteins of interest fused with red fluorescent protein (RFP) and high-throughput screening by merged display analysis, which removes false positive signals from array experiments. To demonstrate large-scale ligand screening for various human proteins of interest, the gene library GLORIA (Gene Library of Osada Laboratory at RIKEN for chemical array analysis) has been established. Using the systematic platform, we detected novel inhibitors of carbonic anhydrase II. We also have shown that this screening method is useful not merely for ligand screening of proteins of interest, but also for gaining insight into structure–affinity relationships (SARs) and for studies of "fragment-based approach."

Traditional fragment-based ligand discoveries have been demonstrated by using several technologies, such as NMR spectroscopy and X-ray crystallography and mass spectrometry. We present initial studies of fragment-based approach to binding assay by using the chemical array format.

Key words: Chemical array, Carbonic anhydrase II, Small molecule, Photochemistry, Bleomycin

1. Introduction

Chemical arrays represent one of the promising and high-throughput approaches to search ligands against proteins of interest. Successful applications of chemical arrays to the discovery of small molecule ligands for a variety of proteins have been reported (1–4). One of the key steps in the technology is immobilization of small molecules on glass slides (5–8). We have developed a nonselective immobilization method that allows various compounds to be immobilized on an array slide in a functional

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_8,

group-independent manner (9). Previous screening using the glass slides where small molecules were immobilized by this immobilization method remained a limitation because it required purified protein (10). Bradner and coworkers reported that mammalian cell lysates that overexpressed target protein could be used for binding assays using chemical arrays (11). We have adopted this system and modified it to create a ligand screening method that enables us to detect the binding ligands of human proteins of interest with a high-throughput manner (12). The success of screening using chemical arrays depends on the choice of target proteins and a number of small molecules to be immobilized on array slides (13). A chemical library, NPDepo (RIKEN Natural Products Depository), was established by our laboratory (14). However, the number of proteins that could be used for chemical array screening was still limited. Hence, we have constructed a human gene library, GLORIA, to accelerate screening on our platform. Our screening method can perform large-scale chemical array screening using the gene library, GLORIA, and a chemical library, NPDepo.

2. Materials

2.1. Cloning of Human cDNAs and Establishment of GLORIA

1. pGEM-T-Easy vector (Promega, Madison, WI).
2. pDsRed-Express-N1 vector (Clontech, Mountain View, CA). pDsRed-Express-N1 is encoded DsRed-Express. DsRed-Express, a variant of *Discosoma* sp. red fluorescent protein (DsRed), forms a homotetramer. This DsRed protein is designated as RFP in this report.
3. Automated sequencer (Applied Biosystems, Foster City, CA).

2.2. Preparation of Mammalian Cell Lysates that Overexpress RFP-fused Proteins

1. RPMI 1640 medium (Invitrogen, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Nichirei Biosciences, Tokyo, Japan).
2. Effectene Transfection Reagent (QIAGEN, Tokyo, Japan).
3. Phosphate-buffered saline (PBS): Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4. Prepare 1× solution before using it.
4. Amount of protein in each lysate was measured with the Bio-Rad protein Assay Reagent (BioRad, Hercules, CA).
5. Typhoon™ 9400 imager (GE Healthcare, Tokyo, Japan).
6. 4× sampling buffer (250 mmol/L Tris-HCL (pH 6.8), 40% glycerol, 8% SDS, 20% 2-mercaptoethanol, and 0.04% bromophenol blue).

2.3. Chemical Array Screening with Cell Lysates

1. Gap cover glass (Matsunami#CG00014, Tokyo, Japan).
2. Hybridization Chamber 5 (TaKaRa#TX711, Tokyo, Japan).
3. Triple Shaker (TAITEC#NR-80, Tokyo, Japan).
4. Spin dryer mini (WAKENYAKU, Kyoto, Japan).
5. GenePix microarray scanner (Amersham Biosciences, Foster City, CA).
6. Photoshop 5.5 software.

2.4. Isothermal Titration Calorimetry and CAII Enzyme Assay

1. MicroCal VP-ITC (MicroCal, Northampton, MA).
2. Carbonic anhydrase and Carbonic anhydrase II (Sigma-Aldrich, Tokyo, Japan).
3. Tris-SO_4 buffer: 50 mM Tris (adjusted to pH 8.4 with H_2SO_4, and DMSO added if necessary).
4. 4-Nitrophenylacetate (Sigma-Aldrich, Tokyo, Japan).
5. Wallac ARVOSX plate reader (PerkinElmer, Turku, Finland).

3. Methods

A human gene library, GLORIA, is designed to be able to express RFP-fused proteins. We have already cloned 100 genes and inserted them into the pDsRed Express-N1 vector. The inserted vectors are stored at –20°C until they are used. Although many proteins are expressed as full-length, some are designed to be expressed as truncated forms, such as membrane proteins (e.g., Bcl-xL and Aggrus). It is important to confirm expression of target proteins when mammalian cell lysates are created.

Our chemical array screening using cell lysates does not require a blocking step. In data analysis of the screening results, we carry out a merged display method that removes false positive signals from array experiments. Although the method makes the visualization of real-hit compounds easier, it is important to demonstrate the detection of positive compounds against the binding proteins, such as rapamycin-FKBP12 and bleomycin-Sh*ble* protein. The reliable positive control on the array slide removes noise and array artifact signals confounding the data analysis.

After hit compounds are obtained from the merged display method, the binding for the target protein is confirmed by a secondary binding assay (e.g., ITC) and by IC_{50} measurements by an enzymatic assay.

3.1. Cloning of Human cDNAs and Establishment of GLORIA

1. Clone target genes from a cDNA library of cultured human cell lines, such as HeLa cells using PCR, and insert the fragments obtained into the pGEM-T-Easy vector.
2. Digest the vectors using several restriction enzymes, and subclone the fragments into the pDsRed-Express-N1 vector (see Notes 1 and 2). Confirm the sequences using the dideoxynucleotide chain termination procedure with an automated sequencer.
3. Store these vectors at –20°C as the gene library GLORIA. The list of the first 100 genes prepared by us is shown in Table 1.

3.2. Preparation of Mammalian Cell Lysates that Overexpress RFP-fused Proteins

1. Maintain HEK293T cells in RPMI-1640 medium supplemented with 10% FCS in a humidified atmosphere containing 5% CO_2. Culture the cells on 60-mm dishes (1×10^6 cells/dish).
2. After 24 h, remove the medium by aspiration and change to fresh ones. Treat the cells using Effectene Transfection Reagent according to Qiagen's protocol with 50 ng plasmid in one dish. Cells are cultured for the next 24–48 h.
3. The cells are washed with PBS to remove remaining medium, harvested, suspended in PBS, and then lysed by sonication. After centrifugation at $20{,}400 \times g$ for 15 min, the supernatant is transferred to a new tube.
4. Measure the protein concentration in each lysate with Bio-Rad protein Assay Reagent. The protein concentration is adjusted to 3–7 mg protein/ml. Overexpression of the RFP-fused protein is confirmed using Typhoon™ 9400 imager and/or by Western blotting (see Note 3). In the case of detections of expression of protein using Typhoon™ 9400 imager, add the 4× sample buffer to the cell lysate described above. Prepare 7.5% SDS-polyacrylamide electrophoresis and load the 10 μl of each sample in a well without boiling. After the samples are separated, the gel is scanned at excitation at 532 nm and emission at 580 nm on a Typhoon™ 9400 imager. Confirm expression of RFP-fused protein by the detection of fluorescent signals on this gel.
5. Use the prepared cell lysates for array experiments immediately. The cell lysates should be kept on ice before they are applied onto the chemical array. According to this instruction, almost 300 μl of cellar extract can be prepared with 3–7 mg/ml total protein concentration from one 60-mm dish.

3.3. Chemical Array Screening with Cell Lysates

1. After preparation of cell lysates that overexpress RFP (for the control slides) and target proteins fused with RFP (for the sample slides), incubate cell lysates (3–7 mg protein/ml) with chemical array slides (for preparation of array slides, see Chapter 2). Reaction volume is set at 40–60 μl for one slide.

Table 1
Partial list of GLORIA

ID no.	Gene names	UniProt no.	ID no.	Gene names	UniProt no.	ID no.	Gene names	UniProt no.	ID no.	Gene names	UniProt no.
1	HPSE	Q9Y251	26	MMP3	P08254	51	ERP27	Q96DN0	76	PARK7	Q99497
2	FKBP1A	P62942	27	ARF6	P62330	52	TXNDC4	Q9BS26	77	CDA	P32320
3	PCBD1	P61457	28	MOCS2	O96033	53	PER1	Q6IN51	78	ASF1A	Q9Y294
4	TIMP1	P01033	29	CDC42EP3	Q9UKI2	54	PSMG2	Q6IAH4	79	ARF1	P84077
5	AGR2	O95994	30	FDPS	P14324	55	NDUFC2	O95298	80	DSTN	P60981
6	RNF34	Q969K3	31	MMP13	P45452	56	TUBA1A	Q71U36	81	MDM2	Q00987
7	PPP6C	O00743	32	TIMP2	P16035	57	TUBG1	P23258	82	DNAJC10	Q8IXB1
8	BCL2L1	Q07817	33	GGPS1	O95749	58	PPME1	Q9Y570	83	PTGS1	P23219
9	MAD2L2	Q9UI95	34	DHFR	P00374	59	ERP29	P30040	84	MED31	Q9Y3C7
10	SFN	P31947	35	BCL2L1[a]	Q07817-2	60	BLMH	Q13867	85	MYCBP	Q99417
11	S100A4	P26447	36	HIG2	Q9Y5L2	61	TGFA	P01135	86	SUB1	P53999
12	S100A10	P60903	37	MAPK1	P28482	62	ATF1	P18846	87	COX7A2L	O14548
13	CDC37	Q16543	38	PCOTH	Q58A44	63	TBCA	Q6FGD7	88	E2F6	O75461
14	AURKB	Q96GD4	39	PPP2CA	P67775	64	PCBD2	Q9H0N5	89	IL1A	P01583
15	CA2	P00918	40	BIRC5	O15392	65	HBXIP	O43504	90	GARS	P41250
16	NME2	P22392	41	PDPN	Q86YL7	66	MMP8	P22894	91	SKP1	P63208
17	IGF2	P01344	42	NME1	P15531	67	MMP10	P09238	92	RBX1	P62877

(continued)

Table 1
(continued)

ID no.	Gene names	UniProt no.	ID no.	Gene names	UniProt no.	ID no.	Gene names	UniProt no.	ID no.	Gene names	UniProt no.
18	EIF4EBP1	Q13541	43	MIRH1	Q75NE6	68	PLAU	P00749	93	AES	Q08117
19	GRB2	P62993	44	YARS	P54577	69	MAP2K1IP1	Q9UHA4	94	REG4	Q9BYZ8
20	PDGFB	P01127	45	WARS	P23381	70	TP53AP1	Q9Y2A0-4	95	NUDT1	P36639
21	HSPB1	P04792	46	IARS	P41252	71	C2orf12	Q8TDM3	96	NEU1	Q99519
22	P4HB	P07237	47	SARS	P49591	72	MAX	P61244	97	TUBB	P07437
23	MAP2K1	Q02750	48	IGF1	Q9NP10	73	DEFA1	P59665	98	MMP9	P14780
24	CENPA	P49450	49	PDIA3	P30101	74	KARS	Q15046	99	PPP1CA	P62136
25	PDIA4	P13667	50	TXNDC12	O95881	75	RAN	P62826	100	JUN	P05412

Gene names and UniProt numbers are based on the UniProt website (http://beta.uniprot.org/)
[a]Splicing variant

2. Cover reaction slides with a gap cover glass and incubated for 1 h at 4°C in Hybridization Chamber 5. Treat control slides and sample slides with cell lysates that overexpress RFP and target proteins fused with RFP, respectively.
3. After incubation, the cover glass is gently slid off and washed with ice-cold PBS 3 times for 1 min using a triple shaker. Dry the glass slides using the mini spin dryer to remove excess liquid and scan at excitation at 532 nm and emission at 575 nm on a GenePix microarray scanner.
4. The GenePix microarray scanner is used to adjust the phase contrast of the two slides between the sample and control to analyze array images.
5. After adjustment of the phase contrasts of the two slides, the sample slides and control slides are colored as red and green, respectively, with Photoshop 5.5 software.
6. Merge the two colored figures into one figure. It is necessary to adjust the position of the compounds on the array using position markers (2 rhodamine spots/144 compounds).
7. Red signals mean hit compounds that bind to the target proteins. On the other hand, yellow signals mean false positive signals that are caused by the binding of ligands to RFP or by autofluorescent signals of the ligand itself. Check the detections of signals of compounds in duplicate on an array slide and distinguish the binding and noise (tiny scratches or patches). Before starting the screening of ligands for target proteins, we recommend one to confirm the detection of known interactions between small molecules and proteins on an array slide by this method. Examples of representative known interactions detected by this method are shown in Fig. 1a–c.
8. Despite duplicate experiments on one slide, it is essential that the array experiments are repeated and done using the cell lysates that overexpress different proteins to confirm whether the obtained signals are specific or not. We detected novel inhibitors of carbonic anhydrase II (Fig. 2a) and various bleomycin derivatives that bind to Sh*ble* protein (Fig. 2b).

3.4. Binding Assay Using ITC and CAII Enzyme Assay

3.4.1. Binding Assay Using ITC

1. We describe here two examples of the binding assay using ITC. ITC experiments are done using MicroCal VP-ITC. In contrast to the chemical array assay, both compounds and proteins are not immobilized onto solid surfaces in ITC experiments. Operation of ITC should be done according to the MicroCal handling protocol.
2. In the case of the carbonic anhydrase II inhibitor assay, ITC experiments are done at 25°C. A solution of carbonic anhydrase is degassed for 3 min and then loaded into the calorimeter syringe. Tris-SO_4 buffer (pH 8.4) containing DMSO 4% (v/v)

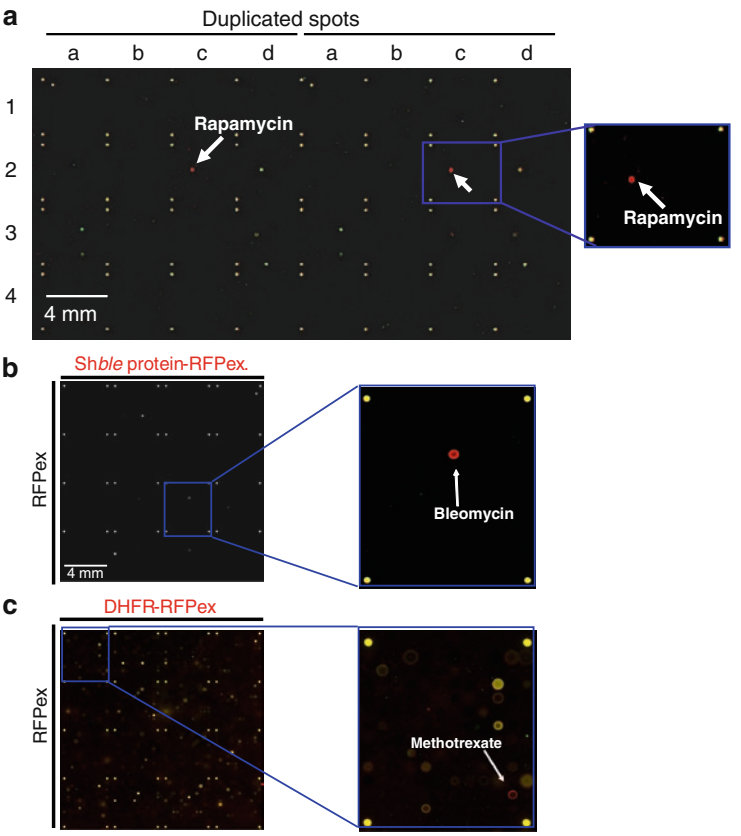
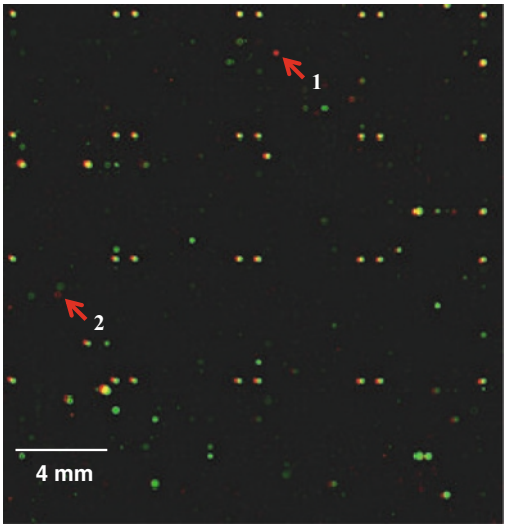


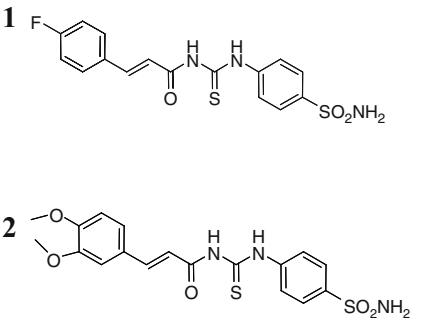
Fig. 1. Identification of rapamycin, bleomycin, and methotrexate. (**a**) Detection of rapamycin used HEK293T cell lysates overexpressing the FKBP12-RFP conjugate. The 2011 small molecules were immobilized on glass slides in duplicate. The 2 rhodamine spots for every 144 compounds were used as position markers. (**b**, **c**) Detection of bleomycin and methotrexate used the cell lysates that overexpressed the Sh*ble*-RFP conjugate (**b**) and DHFR-RFP conjugate (**c**), respectively. All signals of rapamycin, bleomycin, and methotrexate in these experiments were clearly observed as *red spots* and distinguished from the 2011 compounds.

and ligand (5 μM) is degassed for 3 min before use and then titrated with the protein (50 μM) in the same buffer. After an initial dummy injection of 2 μl, injection (10 μl) of the protein solution into the calorimeter cell is done. The resulting titration curves are then processed and fitted with Origin 7 software.

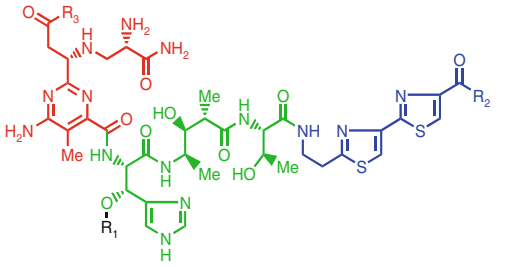
Fig. 2. Fluorescent images by merged display analysis after treatment with cell lysates that expressed RFP-fused CAII (**a**) and Sh*ble* protein (**b**). The HEK293T cell lysates expressing RFP or RFP-fused protein (CAII and Sh*ble* protein) were incubated with the slides, and the slides were briefly washed and scanned. The slides treated by RFP-fused protein (sample) and RFP (control) were colored *red* and *green*, respectively. The merged image is shown. (**a**) The 2011 compounds from NPDepo were immobilized on glass slides in duplicate. Compounds **1** and **2**, indicated as *red arrows*, were identified as novel binders of CAII. (**b**) The 2,000 natural products, including 50 bleomycin derivatives, were immobilized on the glass slide. All of the *red signals* that were observed in the array image were localized to these bleomycin derivatives. The *yellow signals* that were detected were primarily derived from position markers, rhodamine, and fluorescent molecules that were immobilized on the array. Many anthracycline and angucycline derivatives were included in these 2,000 natural products, and they were identified as *yellow spots* in the merged image. Representative bleomycin derivatives **3**–**13** are indicated by *white arrows* and the structures are shown.

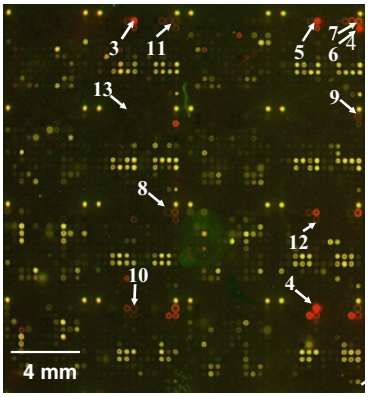
a





b





Compound IDs	R_1	R_2	R_3	Compound Names	On-Chip Categories
3	α-L-gulose-α-D-mannose	$NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3$ - *cyclohexyl*) = *cyclohexyl*)	NH_2	-	high
4	α-L-gulose-α-D-mannose	$NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	NH_2	bleomycin A_6	high
5	α-L-gulose-α-D-mannose	$NH(CH_2)_3NH(CH_2)_4NH_2$	NH_2	bleomycin A_5	high
6	α-L-gulose-α-D-mannose	$NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCOCH_3$	NH_2	-	high
7	α-L-gulose-α-D-mannose	$NH(CH_2)_4NHC(=NH)NH_2$	NH_2	bleomycin B_2	medium
8	α-L-gulose-α-D-mannose	$NHC_6H_4(p\text{-}CH_2N(CH_3)_2)$	NH_2	-	medium
9	α-L-gulose-α-D-mannose	$NH(CH_2)_3SCH_3$	NH_2	bleomycin demethyl A_2	medium
10	α-L-gulose-α-D-mannose	NH_2	NH_2	bleomycin B_1,	medium
11	α-L-gulose-α-D-mannose	OH	NH_2	bleomycinic acid	low
12	H	$NH(CH_2)_4NHC(=NH)NH_2$	NH_2	bleomycin aglycon B_2	low
13	α-L-gulose-α-D-mannose	$NH(CH_2)_4NHC(=NH)NH_2$	OH	*dll*-bleomycin B_2	low

3. In the case of the bleomycin derivatives assay, an ITC experiment is performed at 20°C. A solution of His_6-Sh*ble* protein is degassed for 3 min before being loaded into a calorimeter cell. The solution of the protein (18 μM) in PBS is titrated with ligand solution (200 μM) in PBS that is degassed for 3 min in the same buffer. After an initial dummy injection of 2 μl, 6 μl of ligand solution is injected into the calorimeter cell. The resulting titration curves are then processed and fitted with Origin 7 software.

3.4.2. CAII Enzyme Assay

1. In the measurement of the inhibitory activity of the compounds against CAII, this instruction assumes the use of 4-nitrophenylacetate as substrate, which makes possible detection of the esterase activity of the enzyme (15, 16).
2. Ligands (with increasing concentration) and CAII solution (final 50 nM) are preincubated together for 10 min in 50 mM Tris-SO_4 buffer (pH 8.4; 90 μl).
3. Prepare a 100 mM 4-nitrophenylacetate solution in acetonitrile. Start reaction by the addition of 10 μl of the solution containing 4-nitrophenylacetate (final 10 mM) to the solution prepared above.
4. It is monitored with a Wallac ARVOSX plate reader at 405 nm. The rates of nonenzymatic hydrolysis are subtracted from all observed data. The IC_{50} values are calculated from independent triplicate experiments.

4. Notes

1. In this protocol, we describe the use of RFP (pDsRed-Express-N1 vector) for the assay system. Although other fluorescence proteins are adaptable to our assay format, it should be careful that many small molecules immobilized on the array slides will show fluorescence themselves under conditions of lower excitation and emission filters than the one used to detect RFP.
2. RFP used in this protocol is a tetramer. This RFP tetramer exhibits a higher effective affinity and signal intensity on the array slides compared with the use of an RFP monomer.
3. Preparation of mammalian cell lysates can be adapted for not only FKBP12 and CAII, but also expression of many genes in GLORIA. However, some proteins expressed by this protocol do not come into the supernatant as soluble material.

Therefore, you should confirm whether the RFP-fused protein is in the supernatant or not using a fluorescent scanner and/or Western Blotting before starting array experiments. Not all proteins in GLORIA were examined for retaining the activity of the protein in the cell lysate sample, but it is known that many proteins expressed must be folded in their natural conformation and proper modifications in mammalian cell lysates. If it is possible that you can test the activity of the sample of your target protein, we recommend it.

4. Our chemical array format is useful not merely for screening protein ligands, but also for gaining into structure-affinity relationships (SARs). In the case of bleomycin derivatives, the binding constants that are determined by the ITC experiments correlated well with the binding signal intensities that were observed on the chemical array ($r^2 = 0.663$; Fig. 3).The detailed studies about SARs of bleomycins and Sh*ble* protein have been reported before (17).
5. This chemical array format is also used for the studies of "fragment-based approaches" (18). A solution of fragments is mixed, and the mixture is spotted onto the photoaffinity linker-coated glass slide. When the two fragments that are immobilized as one spot are accessed on the proximal pockets of the target proteins, an increase in binding signal at the mixed spot is observed compared with areas spotted by individual fragments. An example of the result is shown in Fig. 4.

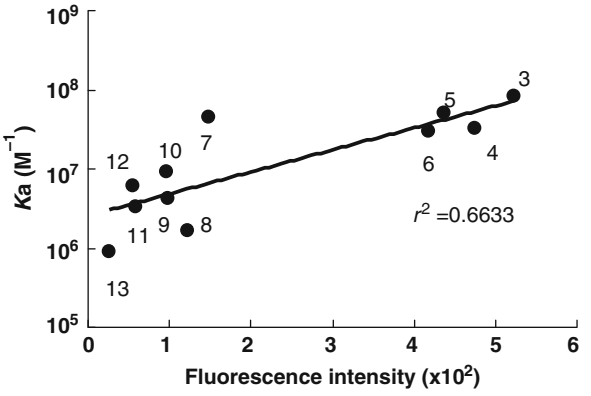


Fig. 3. Correlation between binding constant (*Ka*) obtained from the ITC experiments and fluorescent signal observed on the chemical array. *X*-axis represents the fluorescence intensities of compounds **3**–**13** on the glass slide (Fig. 2b). *Y*-axis represents the *Ka* values calculated by ITC analysis. $r^2 = 0.6633$.

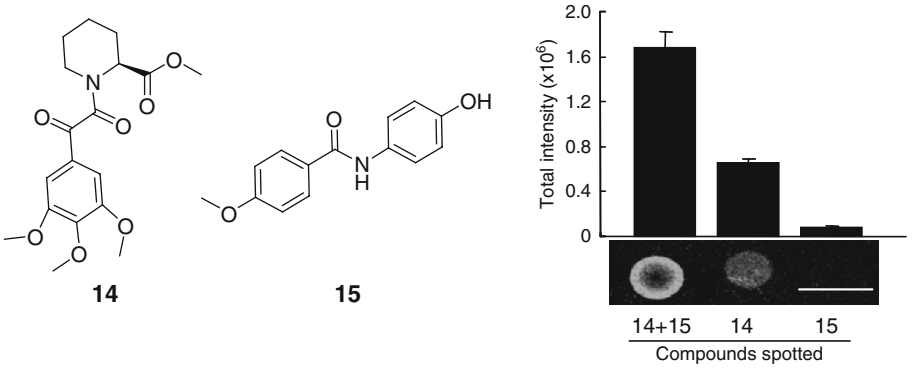


Fig. 4. An example of the detecting fluorescence signals of mixed fragments using our chemical array format. A pipecolinic acid derivative, **14**, has been identified as a ligand for FKBP12 by using NMR, and benzanilide derivative **15** has been obtained as a binder that interacts with the protein at a nearby binding site of **14** (19). Solutions of mixed **14** and **15** (each compound 10 mM) and of independent **15** (10 mM) and **14** (10 mM) were printed on a photoaffinity linker-coated slide. The slide was incubated with HEK293T cell lysates that overexpressed RFP-fused FKBP12. The washed slide was scanned for fluorescence at 532 nm. The total intensity was corrected for background intensity. The *error bars* denote standard deviation over three replicates. An image of the chemical array scanned for fluorescence is shown. An area that was spotted with **14** and **15** exhibited significantly increased fluorescence compared with other areas where they were immobilized individually. *White bar*, 800 μm.

References

1. Kuruvilla, F. G., Shamji, A. F., Sternson, S. M., Hergenrother, P. J., and Schreiber, S. L. (2002) Dissecting glucose signalling with diversity-oriented synthesis and small-molecule microarrays. *Nature* **416**, 653–657.
2. Uttamchandain, M., Walsh, D. P., Yao, S. Q., and Chang, Y.-T. (2005) Small molecule microarrays: recent advances and applications. *Curr. Opin. Chem. Biol.* **9**, 4–13.
3. Koehler, A. N., Shamji, A. F., and Schreiber, S. L. (2003) Discovery of an inhibitor of a transcription factor using small molecule microarrays and diversity-oriented synthesis. *J. Am. Chem. Soc.* **125**, 8420–8421.
4. Uttamchandain, M.,Walsh, D. P., Khersonsky, S. M., Huang, X., and Yao, S. Q. (2004) Microarrays of tagged combinatorial triazine libraries in the discovery of small-molecule ligands of human IgG. *J. Comb. Chem.* **6**, 862–868.
5. Hergenrother, P. J., Depew, K. M., and Schreiber, S. L. (2000) Small-molecule microarrays: covalent attachment and screening of alcohol-containing small molecules on glass slides. *J. Am. Chem. Soc.* **122**, 7849–7850.
6. MacBeath, G., Koehler, A. N., and Schreiber, S. L. (1999) Printing small molecules as microarrays and detecting protein-ligand interactions en masse. *J. Am. Chem. Soc.* **121**, 7967–7968.
7. Park S., and Shin I. (2002) Fabrication of carbohydrate chips for studying protein-carbohydrate interactions. *Angew. Chem. Int. Ed.* **41**, 3180–3182.
8. Barnes-Seeman, D., Park, S. B., Koehler, A. N., and Schreiber, S. L. (2003) Expanding the functional group compatibility of small-molecule microarrays: discovery of novel calmodulin ligands. *Angew. Chem. Int. Ed.* **42**, 2376–2379.
9. Kanoh, N., Kumashiro, S., Simizu, S., Kondoh, Y., Hatakeyama, S., Tashiro, H., and Osada, H. (2003) Immobilization of natural products on glass slides by using a photoaffinity reaction and the detection of protein-small-molecule interactions. *Angew. Chem. Int. Ed.* **42**, 5584–5587.
10. Kanoh, N., Asami, A., Kawatani, M., Honda, K., Kumashiro, S., Takayama, H., Simizu, S., Amemiya, T., Kondoh, Y., Hatakeyama, S., Tsuganezawa, K., Utata, R., Tanaka, A., Yokoyama, S., Tashiro, H., and Osada, H. (2006) Photo-cross-linked small-molecule microarrays as chemical genomic tools for dissecting protein-ligand interactions. *Chem. Asian J.* **1**, 789–797.
11. Bradner, J. E., McPherson, O. M., Mazitschek, R., Barnes-Seeman, D., Shen, J. P., Dhaliwal, J., Stevenson, K. E., Duffner, J. L., Park, S. B., Neuberg, D. S., Nghiem, P., Schreiber, S. L.,

and Koehler, A. N. (2006) A robust small-molecule microarray platform for screening cell lysates. *Chem. Biol.* **13**, 493–504.

12. Miyazaki, I., Simizu, S., Ichimiya, H., Kawatani, M., and Osada, H. (2008) Robust and systematic drug screening method using chemical arrays and the protein library: identification of novel inhibitors of carbonic anhydrase II. *Biosci. Biotechnol. Biochem.* **72**, 2739–2749.
13. He, X. G., Gerona-Navarro, G., Jaffrey, S. R., (2005) Ligand discovery using small molecule microarrays. *J. Pharmacol. Exp. Ther.* **313**, 1–7.
14. Tomiki, T., Saito, T., Ueki, M., Konno, H., Asaoka, T., Suzuki, R., Uramoto, M., Kakeya, H., and Osada, H. (2006) RIKEN natural products encyclopedia (RIKEN NPEdia), a chemical database of RIKEN natural products depository (RIKEN NPDepo). *J. Comp. Aided Chem.* **7**, 157–162.
15. Melkko, S., Scheuermann, J., Dumelin, C. E., and Neri, D. (2004) Encoded self-assembling chemical libraries. *Nat. Biotechnol.*, **22**, 568–574.
16. Poker, Y., and Stone, J. T. (1967) The catalytic versatility of erythrocyte carbonic anhydrase. 3. Kinetic studies of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate. *Biochemistry*, **6**, 668–678.
17. Miyazaki, I., Okumura, H., Simizu, S., Takahashi, Y., Kanoh, N., Muraoka, Y., Nonomura, Y., and Osada, H. (2009) Structure-affinity relationship study of bleomycins and Sh*ble* protein by use of a chemical array. *Chembiochem*, **10**, 845–852.
18. Miyazaki, I., Simizu, S., Ishida, K., and Osada, H. (2009) On-chip fragment-based approach for discovery of high-affinity bivalent inhibitors. *Chembiochem*, **10**, 838–843.
19. Shuker, S. B., Hajduk, P. J., Meadow, R. P., and Fesik, S. W. (1996) Discovering high-affinity ligands for proteins: SAR by NMR. *Science* **274**, 1531–1534.

Chapter 9

Peptide Arrays with a Chip

Alexander Nesterov, Edgar Dörsam, Yun-Chien Cheng, Christopher Schirwitz, Frieder Märkle, Felix Löffler, Kai König, Volker Stadler, Ralf Bischoff, and Frank Breitling

Abstract

Today, lithographic methods enable combinatorial synthesis of >50,000 oligonucleotides per cm^2, an advance that has revolutionized the whole field of genomics. A similar development is expected for the field of proteomics, provided that affordable, very high-density peptide arrays are available. However, peptide arrays lag behind oligonucleotide arrays. This is mainly due to the monomer-by-monomer repeated consecutive coupling of 20 different amino acids associated with lithography, which adds up to an excessive number of coupling cycles. A combinatorial synthesis based on electrically charged solid amino acid particles resolves this problem. A computer chip consecutively addresses the different charged particles to a solid support, where, when completed, the whole layer of solid amino acid particles is melted at once. This frees hitherto immobilized amino acids to couple all 20 different amino acids in one single coupling reaction to the support. The method should allow for the translation of entire genomes into a set of overlapping peptides to be used in proteome research.

Key words: Solid-phase peptide synthesis, Peptide array, Combinatorial synthesis, Amino acid particles, Microchip

1. Introduction

Nature uses a very limited set of only four different bases and 20 different amino acids. These building blocks generate a plethora of different DNA molecules and proteins that together form organelles, cells, and organisms. With respect to sophisticated functions, proteins are the major players in all living organisms. Within ribosomes they replicate themselves and other proteins, polymerases replicate DNA and RNA. As enzymes, proteins perform numerous catalytic reactions, as binding partners they play a central role in inter- and intracellular communication,

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_9, © Springer Science+Business Media, LLC 2010

as transporters they direct many different substances to their destinations, and as antibodies and T-cell receptors they shield us from infections. Also small proteins that are called peptides perform many different functions in the organism. For example, hormones such as insulin or glucagon regulate cell metabolism and defensins function as antibiotics to shield a frog's skin and our gut from infection. The plethora of functions that peptides can perform sparked the interest to get hold on them, and the easiest way to do that is by chemical synthesis (1). Ronald Frank expanded Merrifield's approach by synthesizing many different peptides in parallel (2). His method entailed patterning the 20 different activated amino acid derivatives as small droplets onto a derivatized cellulose sheet. There, consecutive layers react with the free amino groups of the many different growing peptide chains that are linked to the cellulose support during solid-phase synthesis, resulting into a peptide array (3, 4). This SPOT synthesis method, for the first time, made large numbers of peptides available to the scientific community. Over the years, the method has proven to be reliable and widely applicable and thus (5–7), still dominates the field. This is mainly due to a peptide-specific drawback of lithographic synthesis methods that are used to generate high-density oligonucleotide arrays. For the synthesis of peptide arrays, a large number of coupling cycles must be performed, since only a single kind of monomer can be coupled at a time. For example, 20×10 coupling cycles are necessary to synthesize a 10meric peptide array, whereas only 4×10 coupling cycles are needed to generate a 10meric oligonucleotide array (8, 9). This large number of peptide-specific coupling cycles makes it very difficult to avoid the accumulation of unwanted side reactions.

However, the SPOT synthesis method is limited in its ability to produce densities of *in situ* synthesized peptides that exceed 25 peptide spots per cm^2, mainly due to difficulties in handling tiny droplets that tend to evaporate or spread over the array. To overcome these problems, our laboratory recently developed a method for the combinatorial synthesis of peptide arrays that is based on 20 different solid amino acid particles. These triboelectrically charged particles (charged by mild friction) are positioned on a 2D support using electrical fields generated by a laser printer (10) or by a computer chip, where up to 40,000 peptides per cm^2 are synthesized (11) (Fig. 1a). Once positioned, the whole layer of amino acid particles is simultaneously melted to initiate the coupling reaction (Fig. 1b). Washing and deprotection steps complete the cycle, resulting in the combinatorial synthesis of a peptide array, when repeated. This method uses conventional Fmoc chemistry (12) and differs from SPOT synthesis only in the use of a solvent that is solid at room temperature, which enables intermittent immobilization of amino acids within particles. The

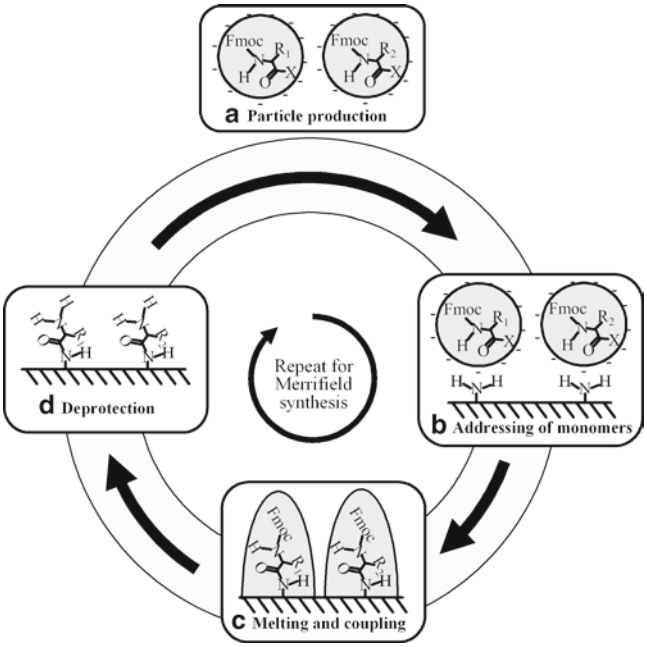


Fig. 1. Particle-based synthesis of peptide arrays. Repetitive cycles generate a peptide array. (**a**) Activated amino acids are embedded in microparticles. (**b**) These are addressed onto a chip by electrical field patterns generated by individual pixel electrodes. (**c**) A whole layer of consecutively addressed amino acid particles is melted at once to induce coupling. (**d**) Residual material from the particles and uncoupled amino acids are washed away and the N-terminal end of the growing peptide chain is deprotected.

number of peptides per cm^2 is only limited by the number of electrodes per cm^2, and by the size of the amino acid particles.

2. Materials

2.1. Production of Amino Acid Particles

1. For the composition of amino acid particles, see also refs. (10, 11), and http:// www.PEPperPRINT.com.
2. The 20 different Fmoc-protected and C-terminally activated amino acid derivatives were from Fluka:

   Fmoc-L-ALanin-OPfp; Fmoc-L-Cys(Trt)-OPfp; Fmoc-L-Asp(OtBu)-OPfp; Fmoc-L-Glu(OtBu)-OPfp; Fmoc-L-Phe-OPfp; Fmoc-dl-Gly-OPfp; Fmoc-L-His(Trt)-OPfp; Fmoc-L-Ile-OPfp; Fmoc-L-Lys(tBoc)-OPfp; Fmoc-L-Leu-OPfp; Fmoc-L-Met-OPfp; Fmoc-L-Asn(Trt)-OPfp; Fmoc-L-Pro-OPfp; Fmoc-L-GLn(Trt)-OPfp; Fmoc-L-Arg(Pbf)-OPfp; Fmoc-L-Ser(OtBu)-OPfp; Fmoc-L-Thr(tBu)-OPfp; Fmoc-L-Val-OPfp; Fmoc-L-Trp-OPfp; Fmoc-L-Tyr(tBu)-OPfp.

3. Acetone p.a.
4. *N*,*N*-Diphenylformamide, DPF (Sigma-Aldrich, Steinheim, Germany).
5. Diphenyl sulfoxide, DPSO (Sigma-Aldrich, Steinheim, Germany).
6. Di-*p*-tolyl sulfoxide, DTSO (Sigma-Aldrich, Steinheim, Germany).
7. Polystyrene (Merck).
8. S-LEC P LT-7552 (Sekisui).
9. Pyrazolone orange (ABCR GmbH).
10. Sodium-di(aqua)-di(2-hydroxy-3-napthoicacido) ferrate (III) (13).
11. Silica particles (Degussa, Aerosil 812, hydrophobic).
12. Distillator (RE111 Rotavapor, Büchi).
13. Freeze dryer (ALPHA I/5, Christ).
14. Air mill (Hosokawa alpine 50AS).
15. Analytical sieves (AS 200 digit, Retsch).
16. Winnower (100 MZR, Hosokawa Alpine AG).
17. Mastersizer (Malvern, type 2000).
18. Q/m meter (Trek, type 210HS-2).
19. Dynamic difference calorimeter (Netzsch, type DSC 204 F1 Phoenix).

2.2. Grafting PEG Surface on the Chip's Surface

1. Mercury vapor lamp (150 W, model TQ 150, Heraeus Noblelight).
2. Pressurized nitrogen.
3. Filtered compressed air that is dried to a low level of humidity (20–30%).
4. 2-Bromo-*N*-(3-triethoxysilyl)propyl isobutyramide (14).
5. Dichloromethane 6. Ethanol p.a.
6. Methanol p.a.
7. 2,2′-Bipyridyl, bipy, 98% (Sigma-Aldrich, Steinheim, Germany).
8. Poly(ethylene glycol) methacrylate, PEGMA, $M_w \sim 360$ g/mol (Sigma-Aldrich, Steinheim, Germany).
9. Copper(I) bromide p.a. (Sigma-Aldrich, Steinheim, Germany).
10. Fmoc-β-alanine, >99% (Fluka).
11. Vacuum desiccator.

2.3. Loading Particles on Microchips

1. Microchips were designed at the Kirchhoff Institute for Physics, University of Heidelberg, and manufactured at the Institute for Microelectronics Stuttgart, IMS CHIPS (http://www.ims-chips.de).
2. Polypropylene tweezers (model K35A, Rubis® Switzerland).
3. Plexiglas aerosol generators for triboelectric charging and amino acid microparticle deposition (Fig. 2, custom-built at the Kirchhoff Institute for Physics, University of Heidelberg; see also ref. (11) accompanying Online Supporting Material).
4. Microscope (model Axiovert 35, Zeiss).
5. Design of circuit boards and bonding with gold wires were done at the Kirchhoff Institute for Physics, University of Heidelberg (http:// www.kip.uni-heidelberg.de). Manufacturing was done at Würth Elektronik GmbH & Co. KG (Niedernhall, Germany).

2.4. Peptide Synthesis

1. The coupling chamber is a 15×6.3×3.3 cm metal box equipped with a lid, gas inlet and outlet valves, and a heat-resistant sealing with two gas valves that allow for degassing and/or controlled nitrogen atmosphere (custom made).
2. The chip on the circuit board is mounted onto a Teflon shield with a chip-size gap (Fig. 3; custom made). Thereby, the circuit -board, extremely sensitive bond wires, and electrical components are protected against harsh chemicals during washing steps, while the chip surface is exposed to chemicals. The chip is mounted or removed from the Teflon shield by four screws.
3. The washing chamber is built of Teflon (Fig. 4; custom made). The protected chip is placed inside the washing chamber and

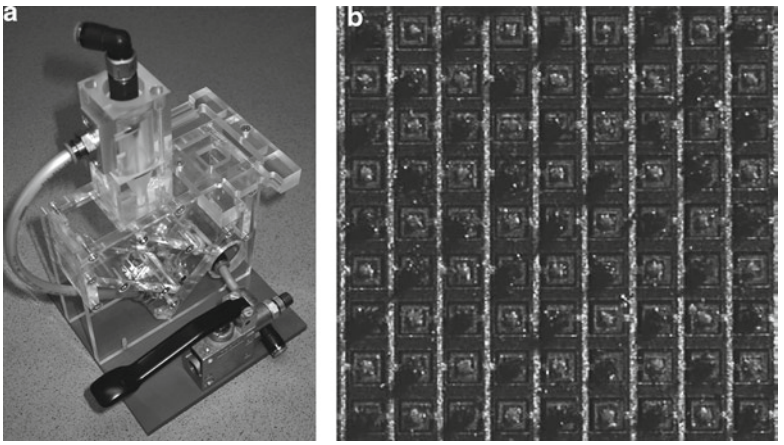


Fig. 2. (**a**) Aerosol chamber. Consecutively deposited microparticles are optionally briefly melted to fix them to the chip. (**b**) For better visualization, colored OKI laser printer toners were employed.

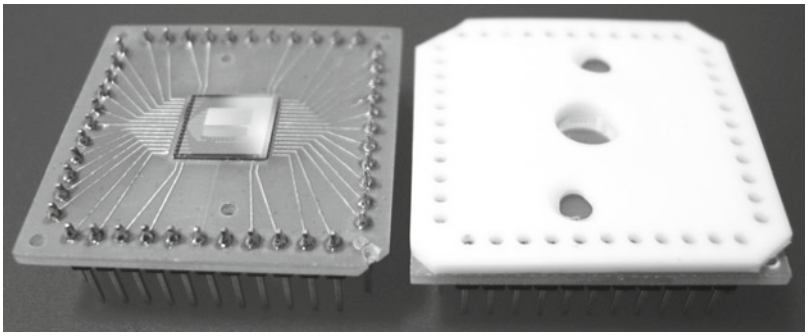


Fig. 3. A Teflon shield is mounted on the circuit board to protect board and bond wires from liquid-phase chemistry during typical washing and deprotection steps in Fmoc peptide synthesis.

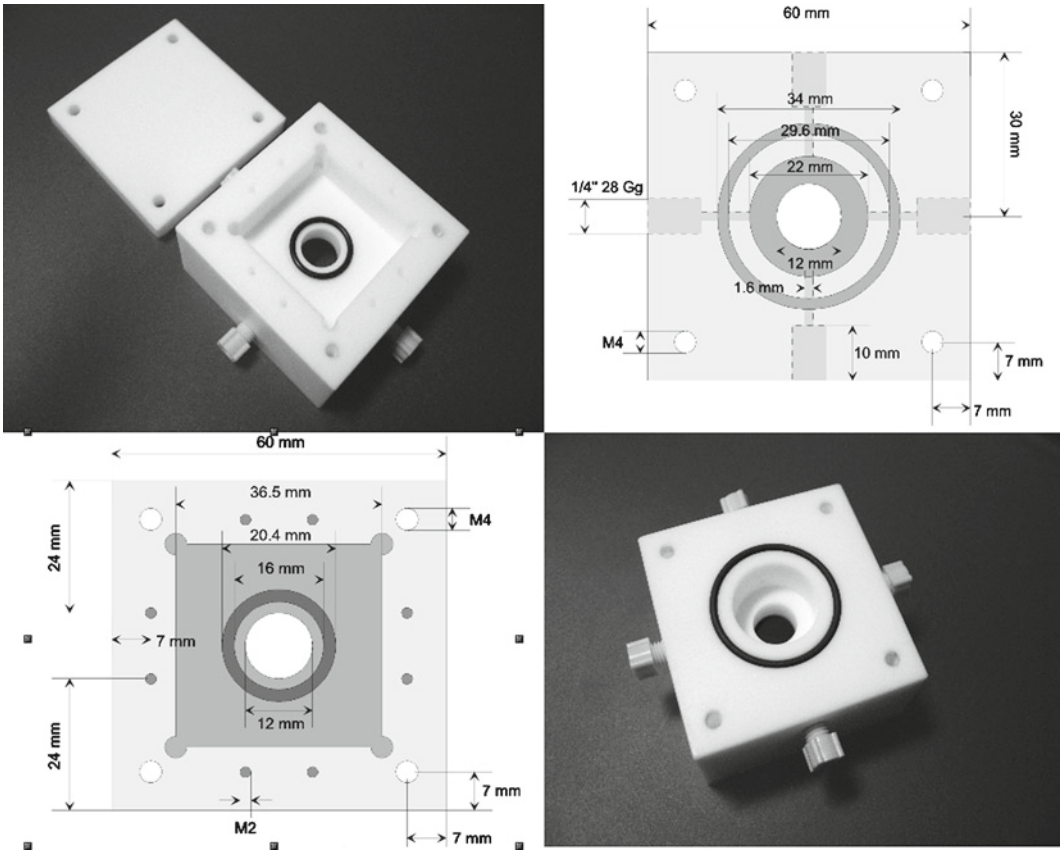


Fig. 4. Construction drawing of a microchip washing chamber made of Teflon.

sealed with an additional Teflon sealing. A stirring bar is placed into the washing chamber above the microchip surface and Teflon shield.

4. *N*,*N*-dimethylformamide, DMF, dried over molecular sieve 0.4 nm (Biosolve BV, Valkenswaard, Netherlands).

5. *N*,*N*′-Diisopropyl carbodiimide, DIC, purum (Carl Roth GmbH, Karlsruhe, Germany).
6. *N*-Methyl-imidazole, NMI, p.a. (Sigma-Aldrich, Steinheim, Germany).
7. Acetic anhydride p.a. (Sigma-Aldrich, Steinheim, Germany).
8. *N*,*N*-Diisopropylethylamine, DIPEA, p.a. (Sigma-Aldrich, Steinheim, Germany).
9. Piperidine ≥99% (Sigma-Aldrich, Steinheim, Germany).
10. Chloroform p.a. (Sigma-Aldrich, Steinheim, Germany).
11. Triisobutylsilane, TIBS, puriss. (Sigma-Aldrich, Steinheim, Germany).
12. Trifluoroacetic acid, TFA, 99% (Acros Organics, Geel, Belgium).

2.5. Staining Peptide Arrays

1. Antibodies: mouse monoclonal anti-FLAG® M2 and rabbit anti-HA (both from Sigma Aldrich, Steinheim, Germany).
2. Secondary antibodies: Alexa Fluor® 647 goat anti-rabbit IgG (heavy + light chains) and Alexa Fluor® 546 goat anti-mouse IgG (H + L) (both from Invitrogen GmbH, Karlsruhe, Germany).
3. TBS: 50 mM Trisma base, 150 mM NaCl, pH 7.4.
4. TBST: 50 mM Trisma base, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.4.
5. TBS–BSA: 50 mM Trisma base, 150 mM NaCl, 1% bovine serum albumine, pH 7.4.
6. Shaker (Duomax 2030, Heidolph GmbH).
7. Magnetic stirrer (model RCT basic IKAMAG safety control, VWR International).
8. GenePix 4000B fluorescence scanner and GenePix Pro 4.0 Microarray Image Analysis software (Molecular Devices).
9. Chip-holder adjusted to the standard scanning format (custom-built metal plate in standard microscopy slide size; equipped with a chip-size gap; tested at Molecular Devices, Munich, Germany).

3. Methods

Our particle-based method employs standard Fmoc solid-phase peptide synthesis chemistry that is extensively described in excellent textbooks and laboratory handbooks (12). Our method adds only one additional trick: C-terminally activated, Fmoc-protected

amino acid derivatives are "frozen at room temperature" within particles. Only when melted, these very reactive chemicals can diffuse and couple to free amino groups on a suitable surface (see Note 1). Therefore, this section mainly deals with the production of amino acid particles, the generation of surfaces suitable for peptide synthesis, and the site-specific loading of particles onto a chip surface.

3.1. Production of Amino Acid Particles

1. Mix together the constituent parts of amino acid particles and solubilize by stirring in 3–4 weight equivalents of acetone (w/w). The ingredients are: Fmoc-protected OPfp -esters of the 20 different amino acids (10% w/w), either *N*,*N*-diphenylformamide (DPF), diphenyl sulfoxide (DPSO), or di-*p*-tolyl sulfoxide (DTSO) as "solid solvent" (25% w/w), resin (60% w/w, either polystyrene or S-LEC P LT-7552), Pyrazolone orange (4% w/w), and sodium-di(aqua)-di(2-hydroxy-3-napthoic acido) ferrate(III) (1% w/w; see Note 2).
2. Remove the bulk of acetone for 20 min at 30°C in a distillator.
3. Remove acetone overnight in a freeze dryer.
4. Store the particle mass under nitrogen in a desiccator for months at room temperature (see Note 1).
5. Premill the particle mass with a rotating scissors mill to yield particles with an average diameter of 100–200 μm (see Note 3).
6. Slowly feed in the resulting particles into an air jet mill. During milling, silica nanoparticles (0.05% w/w) are added (see Note 4).
7. This procedure results in particles with average diameters from 4 to 10 μm (check with microscope).
8. For a narrow size distribution and thus uniform physical properties, remove particles beyond 32 μm with analytical sieves (see Note 5).
9. Remove particles with a diameter <1 μm with a winower (see Note 5).
10. Analyze individual particle batches for narrow size distribution with a mastersizer. Starting with 100 g particle mass, a typical manufacturing run should yield 70 g amino acid particles in the size range between 4 and 20 μm.
11. Analyze particle batches for their triboelectric charge by a Q/m meter. A typical result would yield an electric charge of approx. –4 μC/g of particles (Fig. 5; see Note 5).
12. Analyze particle batches for their melting behavior by dynamic difference calorimetry. The melting point should be between 70 and 75°C.

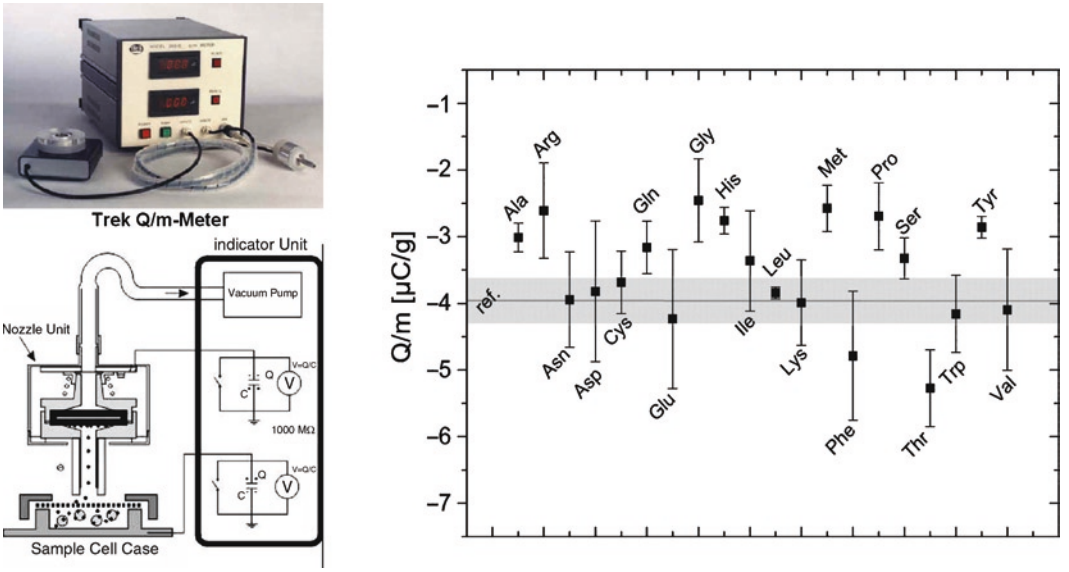


Fig. 5. A Q/m meter is used to determine the amount of charges on the particle surface (*left*). Typical results would yield approx. –4 µC/g particle mass (*right*).

3.2. Grafting the Chip's Surface with a PEG Surface Suitable for Combinatorial Synthesis

1. Handle microchips with polypropylene tweezers to avoid scratching of the surface.
2. In order to silanize the chip surface, place the chip surface 4 cm from the mercury vapor lamp and irradiate for 1 h under atmospheric conditions to remove contaminants by oxidation and to activate the oxide layer on the surface.
3. Turn off the lamp and allow the chip to cool to room temperature.
4. Meanwhile, directly prepare a 10 mM solution of 2-bromo-*N*-(3-triethoxysilyl)propyl isobutyramide (bromine silane) in anhydrous dichloromethane and incubate the chip for at least 6 h to silanize the chip surface with the polymerization initiator.
5. Proceed with this silanization step overnight to achieve a high conversion. Do not incubate longer than 24 h to avoid decomposition of bromine silane.
6. Dilute the reaction solution with an excess of ethanol (twice or thrice the volume of the reaction mixture) and rinse the microchip thoroughly first with ethanol, then with water.
7. Assure appropriate disposal of halogenated organic solvents. Unless otherwise noted, use only Millipore® water (resistance of about 18.2 MΩcm).
8. Wash the chip in ethanol two times for 15 min each and then dry the chip carefully in a stream of compressed air.
9. Place the chip in an oven at 100°C for 1 h to achieve full condensation of the bromine silane with the surface.

10. Remove the chip from the oven and allow it to cool to room temperature.
11. Proceed with graft polymerization **(step 12)** or store the chip at –20°C under nitrogen atmosphere.
12. Dissolve 141 mg 2,2′-bipyridyl (bipy, 0.90 mmol) in 10 ml water/methanol (1:1).
13. Add 5 ml of PEGMA and 64 mg of copper(I) bromide (0.45 mmol). Immediately degas the brown reaction mixture three times and sonicate it for 3 min under nitrogen atmosphere (see Note 6).
14. Remove nondissolved solid copper(I) bromide by filtration through a fritted funnel equipped with a sintered glass disc (fine pore size) under nitrogen atmosphere.
15. Place the silanized chip in a Petri dish inside a desiccator (see Note 7).
16. Add the filtered reaction mixture. The chip should be covered with an excess of solution.
17. Carefully evacuate the desiccator three times and aerate it with nitrogen.
18. Allow polymerization to proceed for 20 h (see Note 8).
19. When the polymerization reaction is finished, open the desiccator.
20. Flush the chip extensively with water inside the Petri dish until all remnants of the polymerization solution are removed.
21. Incubate the chip for 15 min in water.
22. Incubate the chip for 15 min in DMF.
23. Wash the microchip two times for 2 min each with methanol and dry it in a stream of compressed air.
24. Proceed with functionalized PEGMA side chains (step 25) or store the chip at –20°C under nitrogen atmosphere (see Note 9).
25. Preswell the PEGMA-coated chip in anhydrous DMF for 30 min.
26. Prepare a solution of 62.26 mg Fmoc-β-alanine (0.2 m) and 37.2 µl DIC (0.24 m) per ml anhydrous DMF.
27. Stir for 5 min, and then add 37.2 ml NMI (0.4 m).
28. Replace DMF on the microchip with this reaction mixture.
29. Incubate the microchip for 16 h under nitrogen atmosphere at room temperature.
30. Prepare fresh blocking solution 10% (v/v) acetic anhydride, 20% (v/v) DIPEA, and 70% (v/v) DMF immediately before use.
31. Wash the chip three times for 2 min each with DMF.
32. Cover chip with blocking solution to block residual hydroxyl groups.

33. Shake the chip gently overnight in the blocking solution.
34. Remove the blocking solution and wash the chip five times, each time for 2 min with DMF.
35. Cleave the Fmoc protection groups by incubating the chip for 20 min in a solution of 20% (v/v) piperidine in DMF.
36. Wash the chip three times for 2 min with DMF and two times for 2 min with methanol (see Note 10).
37. Dry the chip in a stream of compressed air.
38. Store functionalized chips at –20°C under nitrogen atmosphere (see Note 9).

3.3. Particle Deposition

1. Place the microchip face down in the aerosol generator (Fig. 2).
2. Place aerosol generator into extractor hood, wear dusk masks to avoid inhalation of amino acid particles.
3. Switch a selection of pixel electrodes to voltages of 100 V (vs. grounding) to generate a defined electric field pattern. Take care, high voltage!
4. Initiate the air flow within the aerosol generator to grind the particles against the walls of a smaller cyclone-like cavity (see Note 11).
5. Open the aperture and expose the microchip to the aerosol for 2 s to deposit specific amino acid particles on activated pixels.
6. Check particle pattern with a microscope after each deposition step.
7. Put the chip in a preheated oven at 90°C for 2 min to fix addressed particles on the surface.
8. Repeat particle deposition (Fig. 1b) until all pixel electrodes are covered with different sorts of amino acid particles.

3.4. Solid Phase Peptide Synthesis on Pixel Electrodes

1. Use polypropylene tweezers to transfer the chip into the coupling chamber.
2. Slowly aerate the chamber for 5 min with nitrogen and close valves.
3. Place the coupling chamber into a preheated 90°C oven for 60 min (see Notes 12, 13, and 14).
4. Remove the chip from the oven and let it cool down to room temperature.
5. Mount a Teflon shield by tightening four screws into milled threads (Fig. 3) on the circuit board to protect the board and the bond wires against solvents and reagents (see Note 15).
6. Transfer the microchip into the washing chamber (Fig. 4).
7. Incubate three times (1, 5, and 10 min) with 1 ml of a mixture of 10% (v/v) acetic anhydride, 20% (v/v) DIPEA, and

70% (v/v) DMF to remove residual particle material and, at the same time, block residual amino groups.

8. Wash two times for 5 min each with DMF.
9. Wash two times for 5 min each with chloroform to remove adsorbed silica particles (see Note 16).
10. Incubate the microchip for 30 min in DMF to preswell the polymeric coating.
11. Cleave N-terminal Fmoc-protecting groups by exposing the microchip to 1 ml of 20% (v/v) piperidine in DMF for 20 min.
12. Remove the piperidine/DMF solution and wash five times for 5 min each with DMF.
13. Wash two times for 5 min each with methanol.
14. Dry the microchip in a stream of nitrogen.
15. Remove the chip from the washing chamber and demount the Teflon shield by loosing the four screws.
16. Repeat amino acid particle deposition (preceding paragraph), coupling reaction (this paragraph, steps 1–4), and subsequent washing and deprotection steps (this paragraph, steps 5–15) until the complete peptide array is synthesized.
17. Proceed until the very last coupling step. The number of coupling cycles determines the peptide length (ten cycles of combinatorial synthesis result in an array of 10mers).
18. Leave the microchip from step 16 in the washing chamber in order to cleave the side chain protecting groups.
19. Wash two times for 5 min each with dichloromethane.
20. In the meantime, prepare a mixture of 51% (v/v) trifluoroacetic acid, 44% (v/v) dichloromethane, 3% (v/v) triisobutylsilane (TIBS), and 2% (v/v) water (see Note 17).
21. Remove the dichloromethane and incubate the chip three times for 30 min each in the TFA solution while continuously stirring. Firmly close washing chamber to avoid evaporation of the reagents. Avoid contacting TFA and DMF.
22. Remove the TFA solution.
23. Wash the chip four times for 2 min each with dichloromethane.
24. Wash the chip four times for 2 min each with DMF.
25. Wash the chip two times for 2 min each with ethanol.
26. Dry the chip in a stream of nitrogen.
27. Proceed to immune staining or store the chip at –4°C under nitrogen atmosphere (see Note 9).

3.5. Immunostaining

1. Rehydrate the chip surface by incubation in 1 ml of TBS-T buffer for at least 30 min.

2. Block unspecific binders by incubation in 1 ml of TBS–BSA buffer for 60 min (see Note 18).
3. Dilute mouse anti-FLAG and rabbit anti-HA antibodies each 1:1,000 in TBS-T buffer.
4. Incubate the chip in 1 ml of the antibody solution for 1 h at room temperature. Gently rock the samples.
5. Carefully remove the antibody solution and wash six times for 5 min each in TBS-T with constant gentle shaking.
6. Dilute secondary antibodies Alexa Fluor 647 goat anti-rabbit IgG (H + L) and Alexa Fluor 546 goat anti-mouse IgG (H + L) each 1:1,000 in TBS-T buffer and incubate the chip in this solution for 1 h at room temperature.
7. Wash six times for 5 min each in TBS-T buffer while continuously shaking.
8. Dry the chip in a stream of nitrogen and remove it from the circuit board.
9. Readout fluorescence with an appropriate chip-holder at appropriate wavelengths (532 nm for Alexa Fluor® 546 and 635 nm for Alexa Fluor® 647 dyes, respectively; Fig. 6).

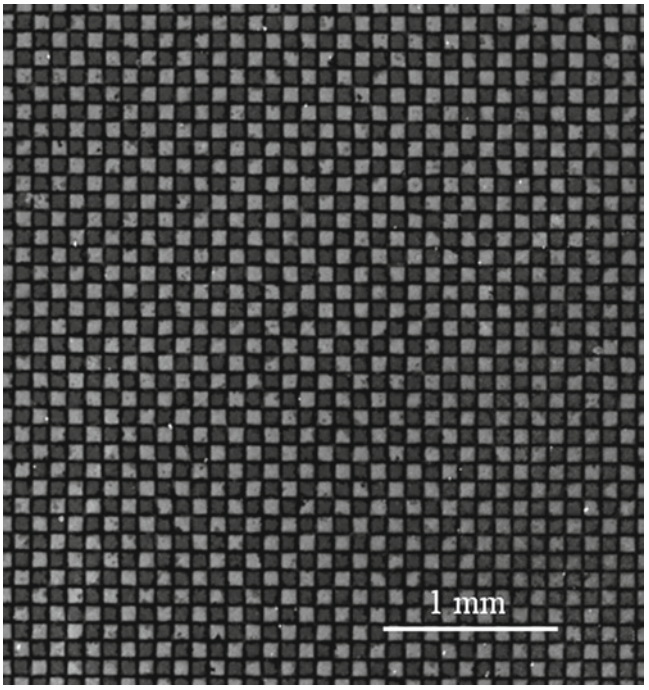


Fig. 6. Particle-based combinatorial synthesis of FLAG® and HA epitopes in a chessboard pattern with a density of 10,000 spots/cm^2. Peptides were stained with rabbit antibodies against FLAG and monoclonal anti-HA mouse antibodies, followed by fluorescently labeled anti-rabbit antibodies and anti-mouse antibodies.

4. Notes

1. Fmoc-protected amino acid derivatives that are chemically activated at their C-terminal end by an OPfp ester are very reactive chemicals that usually have to be frozen immediately at –20°C. However, obviously these chemicals are stabilized within amino acid particles against decay. They can be stored over months at room temperature (10)
2. Triboelectric charging depends on the wall material (PMMA, polymethyl methacrylate in our case), on the particle size, and on the even distribution throughout the particles of "charge control agents," e.g., sodium-di(aqua)-di(2-hydroxy-3-napthoic acido) ferrate(III) (13) that stabilize charges on the surfaces of triboelectrically (particles grinded against the wall) charged particles.
3. During premilling with a rotating scissors mill, particles agglomerate when heated up too much. Let the mill cool down for 15 s in between milling intervals of 15 s.
4. Silica nanoparticles are added to restrain the particle's tendency to agglomerate. The amount of silica nanoparticles needed depends on the size of the amino acid particles.
5. The particle's "jumping behavior" mainly depends on the quotient of electrical charges and the mass of amino acid particles (Q/m). Therefore, particles should have a very narrow size distribution, and they should be uniformly charged.
6. The dissolved copper(I) bromide catalyst is susceptible to oxidation. Discoloration of the solution from brown to green indicates residual oxygen which oxidizes the catalyst and hence disrupts polymerization. Therefore, rapid but careful processing is essential as soon as the copper(I) bromide is added to reaction mixture.
7. Oxygen in the Petri dish or in the desiccator must be avoided.
8. Both concentration of the monomer and polymerization time affect the resulting PEGMA film thickness, whereas film thickness does not noticeably increase after 20 h (15).
9. Chips should not be stored longer than 1 month in a desiccator under nitrogen atmosphere.
10. To provide better accessibility of the growing peptide chains, repeat steps 25–36 two times to consecutively couple three β-alanine residues as linker unit.

11. The geometry of the aerosol chamber evokes an air stream which ensures repeated collision of amino acid particles with the PMMA walls. Thereby, particles are triboelectrically charged and, afterwards, conducted back into the main chamber. Close to the surface of the chip, the particles slow down to a speed of less than ~0.1 m/s which is caused by the chamber geometry at that position. An aperture separates the chip surface from the cyclone airflow to avoid nonspecific deposition of particles due to high particle velocities.
12. The coupling reaction is initiated upon melting of the solid particle matrix. Decay of activated amino acid derivatives inside solid particles is negligible.
13. Coupling times of 60 min at 90°C are not yet optimized. We currently built a custom made hot-water-based oven that should allow reducing these coupling times considerably.
14. Preswelling of the PEG surface augments the repetitive coupling yield from 90% to approx. 96%. However, preswelling also increases unspecific adsorption of particles.
15. Reagents (e.g., DMF, acetic anhydride) used in standard Fmoc synthesis tend to corrode the circuit boards and cause short circuits if they come into contact with bonding wires.
16. Silica nanoparticles are a component part of the amino acid particles. They are removable by washing with chloroform. Eventually, the chloroform step can be omitted in normal cycles. However, at the very end of peptide synthesis, residual silica nanoparticles should be removed by chloroform washing, otherwise leading to unspecific binding signals.
17. Due to the high volatility of dichloromethane, it should be added lastly to the TFA/TIBS/water mixture to avoid evaporation. Caution: TFA is very hygroscopic and corrosive.
18. The PEG surface described in this protocol is very resistant against protein binding. Therefore, no blocking solution is needed to detect the binding, e.g., of the Flag antibody. However, the very same protein resistance blocks low-affinity binders from reaching the surface. In order to also detect low-affinity binders, the surface characteristics should be tuned to a less protein-resistant grade (16).

Acknowledgments

This chapter is dedicated to the memory of Prof. Annemarie Poustka.

References

1. Merrifield, R. B. (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *Journal of the American Chemical Society* **85**, 2149–54.
2. Frank, R. (1992) Spot synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron* **48**, 9217–32.
3. Frank, R. (2002) The SPOT synthesis technique – synthetic peptide arrays on membrane supports – principles and applications. *Journal of Immunological Methods* **267**, 13–26.
4. Hilpert, K., Winkler, D. F. H., and Hancock, R. E. W. (2007) Peptide arrays on cellulose support: SPOT synthesis, a time and cost efficient method for synthesis of large numbers of peptides in a parallel and addressable fashion. *Nature Protocols* **2**, 1333–49.
5. Frank, R. (2002) High density peptide microarrays: emerging tools for functional genomics and proteomics. *Combinatorial Chemistry & High Throughput Screening* **5**, 429–40.
6. Eichler, J. (2005) Synthetic peptide arrays and peptide combinatorial libraries for the exploration of protein–ligand interactions and the design of protein inhibitors. *Combinatorial Chemistry & High Throughput Screening* **8**, 135–43.
7. Min, D-H., and Mrksich, M. (2004) Peptide arrays: towards routine implementation. *Current Opinion in Chemical Biology* **8**, 554–8.
8. Fodor, S. P. A., Read, J. L., Pirrung, M. C., Stryer, L., Lu, A. T., and Solas, D. (1991) Light-directed, spatially addressable parallel chemical synthesis. *Science* **251**, 767–73.
9. Pellois, J. P., Zhou, X., Srivannavit, O., Zhou, T., Gulari, E., and Gao, X. (2002) Individually addressable parallel peptide synthesis on a microchip. *Nature Biotechnology* **20**, 922–6.
10. Stadler, V., Felgenhauer, T., Beyer, M., Fernandez, S., Leibe, K., Güttler, S., Gröning, M., König, K., Torralba, G., Hausmann, M., Lindenstruth, V., Nesterov, A., Block, I., Pipkorn, R., Poustka, A., Bischoff, F. R., and Breitling, F. (2008) Combinatorial synthesis of peptide arrays with a laser printer. *Angewandte Chemie (International ed. in English)* **47**, 7132–35.
11. Beyer, M., Nesterov, A., Block, I., König, K., Felgenhauer, T., Fernandez, S., Leibe, K., Torralba, G., Hausmann, M., Trunk, U., Lindenstruth, V., Bischoff, F. R., Stadler, V., and Breitling, F. (2007) Combinatorial synthesis of peptide arrays onto a microchip. *Science* **318**, 1888.
12. Fmoc solid phase peptide synthesis – A practical approach (Eds.: W. C. Chan, P. D. White), Oxford University Press, Oxford, 2000, pp. 41–76.
13. Kawagishi, Y., Ishida, Y., and Ishikawa, K. (1981) Metal complexes for use in developers for electrostatic images, charge control function. US patent application 4404271A1.
14. Stadler, V., Beyer, M., König, K., Nesterov, A., Torralba, G., Lindenstruth, V., Hausmann, M., Bischoff, F. R., and Breitling, F. (2007) Multifunctional CMOS microchip coatings for protein and peptide arrays. *Journal of Proteome Research* **6**, 3197–202.
15. Beyer, M., Felgenhauer, T., Bischoff, F. R., Breitling, F., and Stadler, V. (2006) A novel glass-slide based peptide array support with high functionality resisting non-specific protein adsorption. *Biomaterials* **27**, 3505–14.
16. Stadler, V., Kirmse, R., Beyer, M., Breitling, F., Ludwig, T. and Bischoff, F. R. (2008) PEGMA/MMA copolymer graftings: Generation, protein resistance, and a hydrophobic domain. *Langmuir* **24**, 8151–57.

Chapter 10

In Situ Chemical Modification of Peptide Microarrays: Characterization by Desorption/Ionization on Silicon Nanowires

Gaëlle Piret, Hervé Drobecq, Rabah Boukherroub, and Oleg Melnyk

Abstract

Peptide microarrays are useful devices for the high throughput study of biomolecular or peptide–cell interactions. Whereas the synthesis of unmodified peptide libraries is an easy task and can be performed at reasonable cost, the synthesis of libraries of modified peptides remains expensive and time consuming. This bottleneck led us to examine the possibility to produce modified peptide microspots by in situ chemical modification of unmodified peptide microspots. The great advantage would be the preparation of a series of complex microarrays (daughter microarrays) starting from an easy-to-make and cost-effective unmodified peptide microarray (parent microarray). One step toward this goal has been presented in the accompanying chapter dealing with the in situ methylation methodology for studying the specificity of antibodies directed toward methylated epitopes. Here we describe the development of a novel desorption/ionization on silicon nanowires mass spectrometry (DIOSiNWs-MS) technique for characterizing the in situ chemical modification of peptides.

Key words: Matrix-free desorption/ionization on silicon nanowires, Mass spectrometry, MALDI-TOF, Peptide microarrays, Methylation, *Mycobacterium tuberculosis*, Heparin-binding hemagglutinin

1. Introduction

In situ modification of unmodified peptide microspots and the preparation of daughter microarrays from a parent unmodified peptide microarray can potentially be applied to various posttranslational modifications. In the accompanying chapter, we have in situ methylated the side-chain of Lys residues by reductive methylation. The chemical process used permitted the formation of mono lysine Lys$(Me)_2$ and dimethyl lysine Lys$(Me)_2$ residues. However, other Lys posttranslational modifications such as acetylation (1), formylation (2), or methylene imine formation (3) can potentially

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_10, © Springer Science+Business Media, LLC 2010

be studied using the in situ modification method. In this context, the development of an analytical method allowing the characterization of the chemical modifications taking place in the peptide microspot is of prime importance for optimizing the in situ chemical modification or for controlling the level of modification.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a soft ionization technique, developed in the late 1980s, for mass analysis of analytes. MALDI allows the analysis of a wide variety of compounds, including polymers, peptides, and proteins (4). However, due to a competitive desorption of parasitic ions from the matrix, it is difficult to detect low molecular weight compounds (<700 m/z). In 1999, Wei et al. reported on the use of porous silicon (PSi) as a substrate for matrix-free desorption/ionization on silicon (DIOS) (5). PSi samples are fabricated by electrochemical etching of crystalline silicon in HF-based solutions. They display good properties of absorption in the UV spectral range and a high surface area. The great interest of DIOS is to permit laser desorption/ionization (LDI) and efficient analysis of small analytes, drugs, explosives, polymers, and hardly soluble analytes in the absence of organic matrix.

We describe here a method based on matrix-free desorption/ionization on silicon nanowires mass spectrometry (DIOSiNWs-MS) for characterizing the in situ chemical modifications occurring inside peptide microspots when a peptide microarray is submitted to chemical reagents. For this, a peptide microarray was prepared on a silicon wafer covered by silicon nanowires (SiNWs), in situ modified by reductive methylation to convert Lys residues into Lys(Me) or Lys$(Me)_2$ residues, and finally analyzed using a MALDI-TOF mass spectrometer (Fig. 1). The DIOSiNWs technique enabled studying the effect of reaction time on the level of methylation of the peptide probes.

2. Materials

2.1. Preparation of SiNWs Substrates for LDI-MS

1. The experiment requires an efficient fume hood.
2. P-type <100> crystalline silicon wafers (Siltronix) with a resistivity of 0.01 Ω cm (typically 10 mm × 20 mm × 380 µm).
3. VLSI-grade (very large-scale integration-grade) sulfuric acid, 96% (H_2SO_4).
4. VLSI-grade hydrogen peroxide, 30% (H_2O_2).
5. VLSI-grade hydrofluoric acid, 50% (HF).
6. ACS grade (>99&, Sigma-Aldrich) silver nitrate 0.1 N ($AgNO_3$).
7. VLSI-grade nitric acid, 65% (HNO_3).
8. VLSI-grade hydrochloric acid, 37% (HCl).
9. Acetone.

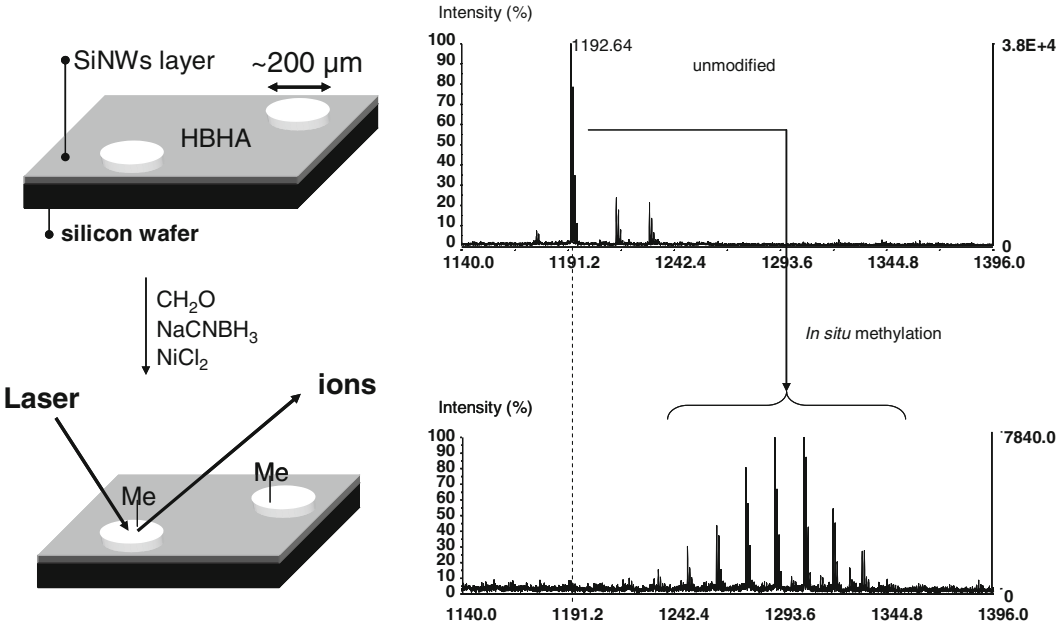


Fig. 1. Application of the DIOSiNWs-MS technique for characterizing the in situ methylation of peptide microspots. HBHA peptide H-LPKKAAPAKKAA-NH_2 was printed on a silicon wafer substrate covered with a SiNWs layer, and in situ methylated with formaldehyde/$NaCNBH_3$/$NiCl_2$. Analysis of peptide microspots by LDI-MS permitted to characterize the in situ chemical modification of HBHA peptide.

10. Isopropyl alcohol (*i*PrOH).
11. Dichloromethane (CH_2Cl_2).
12. Octyldimethylchlorosilane (ODMCS).
13. UV-O Cleaner, Jelight Company Inc., 4 mW/cm^2 at 220 nm.
14. Plas-Labs nitrogen glove box.
15. Goniometer system (DIGIDROP, GBX, France) for measuring the contact angles.

2.2. Printing of Peptide Probes

1. Peptide probes.
2. 0.01 M Sodium phosphate buffer pH 7.2 containing 150 mM NaCl (PBS).
3. V-bottomed 384-well microtiter plates (ABgene, Surrey, UK, AB-1058).
4. Noncontact piezoelectric microarrayer (BCA1, Perkin Elmer, MA, USA).

2.3. In Situ Methylation

1. Prepare solution 1: First, prepare a 16 mg/mL $NiCl_2$ solution in HEPES 0.1 M pH 7.5 buffer. Then, dissolve $NaCNBH_3$ to 15 mg/mL in this solution.
2. Prepare solution 2: mix 1 volume of formaldehyde (36% w/v aqueous solution) with 999 volumes of HEPES 0.1 M pH 7.5 buffer.

3. Prepare solution 3: mix 1 volume of solution 2 with 9 volumes of solution 1 (final formaldehyde concentration 0.036 mg/mL).
4. Microscope glass slide.
5. Corning incubation chamber (ref 732-5503, VWR).

2.4. Laser Desorption/Ionization and Mass Spectrometry Analysis

1. MALDI-TOF mass spectrometer equipped with delayed extraction and operating with a pulsed N_2 laser at 337 nm (Voyager-DE™ STR Biospectrometry Workstation, Applied Biosystems).
2. Stainless steel Voyager™ MALDI sample plate with surface indent to be used with gel membranes (Part No. V700698, Applied Biosystems).
3. Standard carbon adhesive tabs, diameter 25 mm (EUROMEDEX).
4. Micropipette (1–20 µL).
5. Calibration mixture (Sequazyme™ Mass Standards Kit, Part No. P2-3143-00, Applied Biosystems).
6. 15 mg/mL 2,5-Dihydroxybenzoic acid (DHB) solution in acetone.

3. Methods

The preparation of peptide microarrays on SiNWs substrates and the use of matrix-free LDI-MS for characterizing the in situ chemical modification of a peptide microspot are illustrated with nHBHA 159–170 peptide H-LPKKAAPAKKAA-NH_2 (see Note 1 and also chapter 11). First, a DIOS substrate made of SiNWs on a silicon wafer was prepared by etching crystalline silicon in HF/$AgNO_3$ aqueous solution (6, 7). The length (2.5 µm) and diameter (10–100 nm) of SiNWs were optimized to reach an optimal sensitivity in LDI-MS detection. The SiNWs surface was then silanized with ODMCS in hexane (see Note 2) (6, 8). The peptide was dissolved in ammonium citrate and printed on the silanized SiNWs substrate using a noncontact microarrayer. In situ methylation was performed as described in the chapter 11 in the presence of formaldehyde, cyanoborohydride, and nickel chloride. However, the concentration of nickel chloride was lowered as compared to the work performed with microscope glass slide substrates to optimize the signal-to-noise ratio of the mass spectra (see Note 3).

The data presented show that the SiNWs substrates allow the monitoring of the in situ modification of the peptide microspots using matrix-free LDI-MS (Fig. 2). Formation of the permethylated

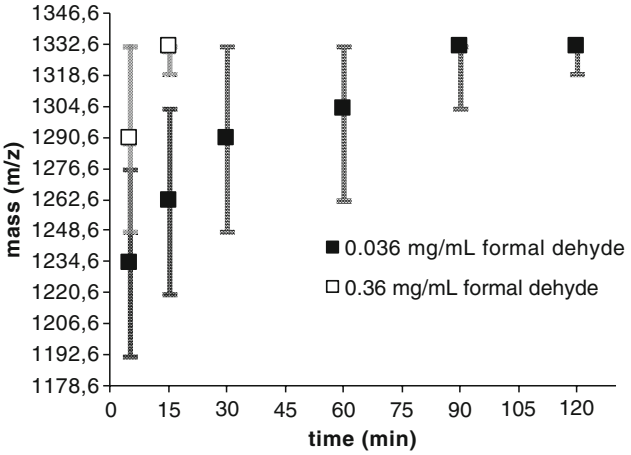


Fig. 2. LDI-MS analysis after 5, 15, 30, 60, 90, and 120 min of in situ reductive methylation of HBHA peptide microspots. The spectra were acquired in positive ion mode (accelerating potential 20 kV, grid voltage 73%, extraction delay time 200 ns, reflector mode). Each spectrum is the result of ~50 averaged laser pulses. The *square* indicates the peak of highest intensity; the bar indicates the range of ions detected. Unmodified HBHA peptide $[M+H]^+$ monoisotopic calcd. 1,192.78, found 1,192.6, permethylated HBHA peptide $[M+H]^+$ monoisotopic calcd. 1,332.95, found 1,332.6.

HBHA peptide is detected after only 30 min of reaction. It is the main compound detected after 120 min. The experimental conditions for the in situ methylation reaction (reaction time, concentration of the reagents) can be varied to obtain different methylation patterns.

3.1. Preparation of Silicon Nanowires for LDI-MS (See Notes 4 *and* 5*)*

1. Degrease the silicon wafer in acetone and *i*PrOH, rinse with deionized water.
2. Clean the substrate in a piranha solution (H_2SO_4/H_2O_2: 3/1 by vol) for 20 min at 80°C, and then rinse copiously with deionized water.
3. Immerse the substrate in an aqueous $HF/AgNO_3$ (5.25/0.02 M) solution at 55°C for 10 min (see Note 6).
4. Rinse copiously with deionized water and immerse the resulting substrates in an aqueous solution of $HCl/HNO_3/H_2O$: 1/1/1 by vol at room temperature overnight (see Note 7).
5. Clean the SiNWs surface in acetone and *i*PrOH, rinse with deionized water.
6. Apply an UV/ozone – treatment for 30 min to remove any organic contaminants on the surface and to generate surface hydroxyl groups.
7. Immerse the substrate in a 1 mM ODMCS solution in hexane for 3 h at room temperature in a dry nitrogen purged glove box.

8. Rinse the substrate with CH_2Cl_2 and *i*PrOH, and dry under a gentle stream of nitrogen.
9. Characterize the substrate with a goniometer (water contact angles were measured using deionized water in ambient atmosphere at room temperature, volume of the drops 1 μL). The ODMCS-terminated SiNWs surface displays a hydrophobic character with a water contact angle of $105 \pm 2°$.

3.2. Microarray Printing

1. Dissolve the peptides to 1 mM in a 1 mM ammonium citrate aqueous solution.
2. Place 20 μL of each peptide solution in a V-bottomed 384-well microtiter plate.
3. Print with a noncontact microarrayer (one drop, 330 pL). Separate the microspots by 1,200 μm.

3.3. In Situ Methylation

1. Place the SiNWs substrate on a microscope glass slide.
2. Deposit solution 3 on the SiNWs substrate (35 μL per microarray, see Note 8).
3. Insert the microscope glass slide together with the substrate inside the incubation chamber. Close the incubation chamber. The liquid must be trapped between the SiNWs substrate and the top plate of the incubation chamber and cover all the microarray pattern.
4. Remove the SiNWs substrate from the incubation chamber and rinse twice with 1 mM aqueous ammonium citrate.
5. Dry the microarray under a stream of air.
6. Use the methylated microarray immediately in the next step.

3.4. LDI-MS Analysis

1. Paste the SiNWs substrate on the stainless plate with carbon adhesive tabs.
2. Deposit manually with a micropipette 1 μL of DHB solution in acetone near the microarray pattern.
3. Deposit manually with a micropipette 1 μL of the calibration mixture (typically 1 μM of each peptide from the calibration kit) near the microarray pattern.
4. Insert the sample in the MALDI-TOF mass spectrometer (see Note 9).
5. Use the DHB spot to determine where the laser beam hits the substrate (see Note 10).
6. Acquire positive ion mass spectra in reflector mode.
7. Calibrate.

4. Notes

1. *Mycobacterium tuberculosis* heparin-binding hemagglutinin (HBHA) is a surface-associated virulence factor and a powerful diagnostic and protective antigen (9–11). Native HBHA (nHBHA) contains 13 mono- or dimethyl lysines in its C-terminal 159–198 domain, resulting in a complex methylation pattern (12, 13). Three lysines within HBHA peptide used in this study (H-$L^{159}PK^{161}K^{162}AAPAK^{167}K^{168}AA$-$NH_2$) are mono- or dimethylated in the native protein (K^{162}, K^{167}, and K^{168}). The in situ methylation procedure leads to the methylation of all free amino groups. Consequently, the N-terminal amino group of L^{159} and the side-chain amino group of K^{161} are also methylated. Thus, up to ten methyl groups can be introduced on the peptide.
2. Silanization of SiNWs with ODMCS was required to reach acceptable peptide microspot sizes and to limit the droplet spreading. The spreading of water droplets on unmodified super hydrophilic SiNWs affects the desorption/ionization efficiency and the sensitivity of detection. Silanization with 3-aminopropyltrimethoxysilane failed to give detectable ion peaks for the studied peptides. On the other hand, silanization of the SiNWs surface with other silanes such as octadecyltrichlorosilane (OTS) or perfluorodecyltrichlorosilane (FDTS) leads to the formation of superhydrophobic SiNWs surfaces (water contact angle ~160°). A water droplet deposited on such surfaces has the tendency to roll of the surface, a property that precludes their use for microarray preparation.
3. The role of nickel(II) ions is to capture cyanide ions released in the methylation mixture, and thus to avoid the formation of cyanomethylation side-products during the reductive methylation. However, too high concentrations of nickel chloride alter the S/N ratio of the MS analysis.
4. The mixture H_2SO_4/H_2O_2 (piranha) solution is a strong oxidant. It reacts violently with organic materials. It can cause severe skin burns. It must be handled with extreme care in a well-ventilated fume hood while wearing appropriate chemical safety protection.
5. HF is a hazardous acid which can result in serious tissue damage if burns were not appropriately treated. Etching of silicon should be performed in a well-ventilated fume hood with appropriate safety considerations: face shield and double-layered nitrile gloves.
6. The silicon substrate is oxidized by silver ions into silicon oxide, which in turn is removed by HF.

7. Oxidation of silicon by silver ions results in the deposition of metallic silver on the substrate. This step allows removing the silver nanoparticles and dendrites deposited on the SiNWs during the chemical etching in aqueous $HF/AgNO_3$.
8. These solutions must be prepared just before the in situ methylation step. The kinetics of the in situ reductive methylation depends on the concentration of the reagents and of formaldehyde in particular. The methylation pattern obtained using a final formaldehyde concentration of 0.036 mg/mL and 120 min of reaction (Fig. 2) or a final formaldehyde concentration of 0.36 mg/mL and 15 min of reaction was similar.
9. The thickness of the sample plate (SiNWs substrate on the stainless steel) must be checked to avoid a blockade in the spectrometer.
10. The thickness of the sample plate (stainless steel + carbon adhesive + SiNWs substrate) is usually different from those of a standard MALDI-TOF sample plate. Because the laser beam hits the sample plate with an angle of 45°, the area illuminated by the laser changes with sample thickness. This must be checked carefully before recording the MS spectra.

Acknowledgments

We gratefully acknowledge the financial support from CNRS, Université de Lille Nord de France, Institut Pasteur de Lille, IFR 142, Région Nord Pas de Calais, the European Community (FEDER), and from Cancéropôle Nord-Ouest. This research was performed using the Chemistry Systems Biology platform (http://csb.ibl.fr). GP thanks the DGA (Direction Générale de l'Armement) for support with a PhD scholarship.

References

1. Yang, X. J., and Seto, E. (2008) Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol. Cell* **31**, 449–61.
2. Wisniewski, J. R., Zougman, A., and Mann, M. (2008) Nepsilon-formylation of lysine is a widespread post-translational modification of nuclear proteins occurring at residues involved in regulation of chromatin function. *Nucleic Acids Res.* **36**, 570–7.
3. Floor, E., Maples, A. M., Rankin, C. A., Yaganti, V. M., Shank, S. S., Nichols, G. S., O'Laughlin, M., Galeva, N. A., and Williams, T. D. (2006) A one-carbon modification of protein lysine associated with elevated oxidative stress in human substantia nigra. *J. Neurochem.* **97**, 504–14.
4. Karas, M., and Hillenkamp, F. (1988) Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal. Chem.* **60**, 2299–301.
5. Wei, J., Buriak, J. M., and Siuzdak, G. (1999) Desorption-ionization mass spectrometry on porous silicon. *Nature* **399**, 243–6.
6. Piret, G., Coffinier, Y., Roux, C., Melnyk, O., and Boukherroub, R. (2008) Biomolecule and nanoparticle transfer on patterned and

heterogeneously wetted superhydrophobic silicon nanowire surfaces. *Langmuir* **24**, 1670–2.

7. Douani, R., Hadjersi, T., Boukherroub, R., Adour, L., and Manseri, A. (2008). Formation of aligned silicon-nanowire on silicon in aqueous $HF/(AgNO_3 + Na_2S_2O_8)$ solution. *Appl. Surf. Sci.* **254**, 7219–22
8. Coffinier, Y., Janel, S., Addad, A., Blossey, R., Gengembre, L., Payen, E., and Boukherroub, R. (2007) Preparation of superhydrophobic silicon nanowires surfaces. *Langmuir* **23**, 1608–11.
9. Locht, C., Hougardy, J. M., Rouanet, C., Place, S., and Mascart, F. (2006) Heparin-binding hemagglutinin, from an extrapulmonary dissemination factor to a powerful diagnostic and protective antigen against tuberculosis. *Tuberculosis (Edinb)* **86**, 303–9.
10. Menozzi, F. D., Bischoff, R., Fort, E., Brennan, M. J., and Locht, C. (1998) Molecular characterization of the mycobacterial heparin-binding hemagglutinin, a mycobacterial adhesin. *Proc. Natl Acad. Sci. U S A* **95**, 12625–30.
11. Menozzi, F. D., Rouse, J. H., Alavi, M., Laude-Sharp, M., Muller, J., Bischoff, R., Brennan, M. J., and Locht, C. (1996) Identification of a heparin-binding hemagglutinin present in mycobacteria. *J. Exp. Med.* **184**, 993–1001.
12. Pethe, K., Bifani, P., Drobecq, H., Sergheraert, C., Debrie, A. S., Locht, C., and Menozzi, F. D. (2002) Mycobacterial heparin-binding hemagglutinin and laminin-binding protein share antigenic methyllysines that confer resistance to proteolysis. *Proc. Natl Acad. Sci. U S A* **99**, 10759–64.
13. Temmerman, S., Pethe, K., Parra, M., Alonso, S., Rouanet, C., Pickett, T., Drowart, A., Debrie, A. S., Delogu, G., Menozzi, F. D., Sergheraert, C., Brennan, M. J., Mascart, F., and Locht, C. (2004) Methylation-dependent T cell immunity to *Mycobacterium tuberculosis* heparin-binding hemagglutinin. *Nat. Med.* **10**, 935–41.

Chapter 11

In Situ Chemical Modification of Peptide Microarrays: Application to the Study of the Antibody Responses to Methylated Antigens

Rémi Desmet, Eric Diesis, Hervé Drobecq, Carine Rouanet, Karim Chemlal, Anne-Sophie Debrie, Jean-Michel Hougardy, Françoise Mascart, Camille Locht, and Oleg Melnyk

Abstract

Peptide microarrays are useful tools for characterizing the humoral response against methylated antigens. They are usually prepared by printing unmodified and methylated peptides on substrates such as functionalized microscope glass slides. The preferential capture of antibodies by methylated peptides suggests the specific recognition of methylated epitopes. However, unmodified peptide epitopes can be masked due to their interaction with the substrate. The accessibility of unmodified peptides and thus the specificity of the recognition of methylated peptide epitopes can be probed using the in situ methylation procedure described here. Alternately, the in situ methylation of peptide microarrays allows probing the presence of antibodies directed toward methylated epitopes starting from easy-to-make and cost-effective unmodified peptide libraries. In situ methylation was performed using formaldehyde in the presence of sodium cyanoborohydride and nickel chloride. This chemical procedure converts lysine residues into mono- or dimethyl lysines.

Key words: Peptide microarrays, Microscope glass slides, Methylation, Antibody, *Mycobacterium tuberculosis*, Heparin-binding hemagglutinin (HBHA), Fluorescence

1. Introduction

Peptide microarrays are useful devices for the high throughput study of biomolecular or peptide–cell interactions. In particular, peptide microarrays are often used for the detection of antibodies (1–8). The parallel detection of antibodies in biological samples has a wide range of potential applications in the diagnosis of allergies, autoimmune and infectious diseases as well as in epitope

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_11, © Springer Science+Business Media, LLC 2010

mapping studies and the development of vaccines (9–15). Here we used novel peptide microarray tools for characterizing the humoral response against methylated peptides derived from *Mycobacterium tuberculosis* heparin-binding hemagglutinin (HBHA), a surface-associated virulence factor, and a powerful diagnostic and protective antigen (for a review, see ref. (16)).

Native HBHA (nHBHA) contains 13 mono- or dimethyl lysines in its C-terminal domain, resulting in a complex methylation pattern (17, 18). To analyze methylation-specific antibody responses, methylated or unmodified peptides derived from this domain were synthesized and microarrayed onto glass slides together with control peptides and native methylated HBHA (nHBHA). Sera from nHBHA-immunized mice reacted with some methylated peptides but not with their unmodified analogs, suggesting that methylation is necessary for antibody recognition. However, the low signal strength obtained in the absence of methylation for some peptides might be due to a reduced accessibility of unmodified epitopes toward antibodies relative to methylated ones. Alternately, this difference could be due to the preferential desorption of unmodified peptides from the substrate.

We describe here a simple method for probing the accessibility of lysine residues within unmodified peptides, based on the in situ methylation of peptide microspots (Fig. 1). A parent microarray composed of unmodified and methylated peptide

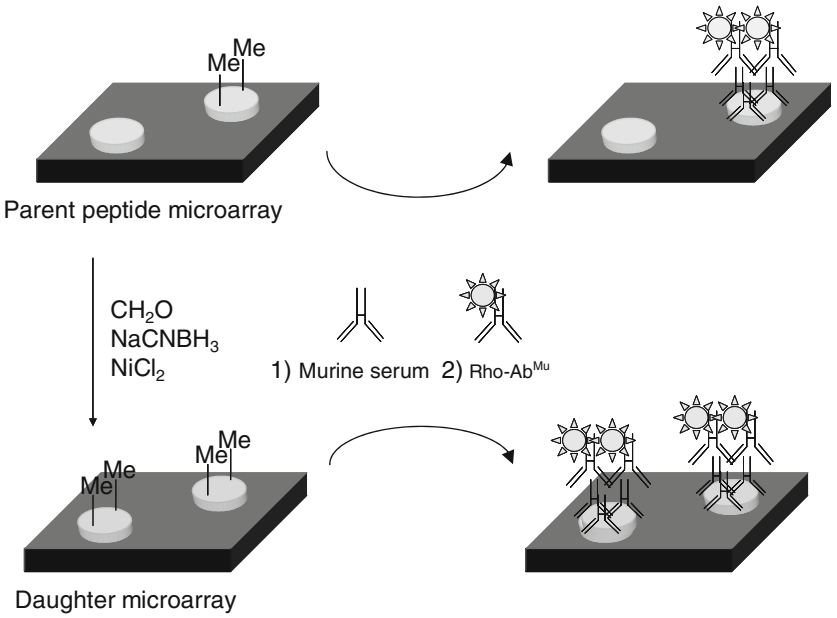


Fig. 1. Strategy used to document the accessibility of Lys residues in a microarray experiment and the specificity of antibody binding to methylated epitopes.

probes was methylated to give a daughter microarray. Both microarrays were used for capturing antibodies present in the sera from nHBHA-immunized mice. The detection of captured antibodies was performed using tetramethylrhodamine-labeled secondary antibodies. The capture of antibodies by the in situ methylated peptide microspots within the daughter microarray but not within the parent microarray shows the accessibility of Lys residues toward the aqueous medium and the specificity of antibodies for methylated epitopes.

Another interesting application of the in situ methylation procedure described here is the possibility to probe the presence of antibodies directed toward methylated epitopes starting from easy-to-make and cost-effective unmodified peptide libraries and standard microarray techniques. Peptide microarrays were methylated by reductive alkylation with formaldehyde in the presence of sodium cyanoborohydride and nickel chloride, a procedure which allows the incorporation of up to two methyl groups on each primary amines within the peptide probes (19). Thus, the method is restricted to the study of mono- or dimethyl lysines.

2. Materials

2.1. Preparation of Amine-Functionalized Microscope Glass Slides

1. The experiment requires an efficient fume hood (see Note 1).
2. Microscope glass slides.
3. Glass staining dishes, a Teflon® staining dish; and a glass slide rack for 20 slides (the removable glass slide rack has an open bottom, which allows for rapid filling and draining).
4. Magnetic stirring bars and a magnetic stirrer.
5. Glass dessicator and a vacuum pump.
6. A Teflon® pot (1 L, ref 213-0242, VWR).
7. Sulfuric acid.
8. Hydrogen peroxide (H_2O_2, 30% aqueous solution).
9. 3-Aminopropyltrimethoxysilane.
10. Methanol (MeOH, Carlo Erba).
11. Deionized water (see Note 2).
12. Oven.
13. Goniometer (GBX, Romans sur Isère, France).

2.2. Printing

1. Peptide and protein probes.
2. 0.01 M sodium phosphate buffer pH 7.2 containing 150 mM NaCl (PBS).

3. V-bottomed 384-well microtiter plates (ABgene, Surrey, UK, AB-1058).
4. Microarrayer (see Note 3).

2.3. In Situ Methylation

1. Prepare solution 1: dissolve $NiCl_2$ to 47 mg/mL in HEPES 0.1 M pH 7.5 buffer. Then, dissolve $NaCNBH_3$ to 15 mg/mL in this solution (see Note 4).
2. Prepare solution 2: mix 1 volume of formaldehyde (36% w/v aqueous solution, ACROS Organics) with 9 volumes of HEPES 0.1 M pH 7.5 buffer (see Note 4).
3. Prepare solution 3: mix 1 volume of solution 2 with 9 volumes of solution 1 (see Note 4).
4. Cover glasses.

2.4. Incubations and Scanning

1. 0.01 M sodium phosphate buffer pH 7.2 containing 150 mM NaCl (PBS).
2. Prepare PBS-A buffer: 0.05% v/v Tween® 20 in PBS.
3. Prepare PBS-B buffer: 2% w/v bovine serum albumin (BSA) in PBS.
4. Primary antibodies: anti-HA murine antibody (HA.11 from ascites, Eurogentec, Belgium), anti-FLAG® M2 murine antibody (F 1804, affinity purified, Sigma, MI, USA), mouse IgG (ref 015-000-003, Jackson Immunoresearch, PA, USA), sera from nHBHA-immunized mice or naïve mice.
5. Tetramethylrhodamine-labeled goat anti-murine antibody (Sigma, France).
6. Absolute ethanol.
7. Cover glasses.
8. Oven.
9. Laminar flow hood.
10. Fluorescence microarray scanner (532 nm, Innopsys scanner, France) and Mapix® image analysis software (Innopsys, France).

3. Methods

nHBHA 1–4 peptides used in this study are derived from the C-terminal 158–198 domain of nHBHA, which presents a complex methylation pattern due to the presence of 13 mono- or dimethyl lysine residues (Table 1) (see Note 5). Peptides HBHA 1–4 were permethylated in aqueous solution in the presence of an excess of $NaCNBH_3$/formaldehyde/$NiCl_2$ to give

Table 1
Peptides and proteins used in this study

Peptide	Description
HBHA1	H-L^{158}PK^{160}KAAPAK^{166}K^{167}AAPAK^{172}K^{173}AAPAK^{178}K^{179}AA^{181}-NH_2
HBHA1 M	$(Me)_2$-L^{158}P*KK*AAPA*KK*AAPA*KK*AAPA*KK*AA-NH_2
HBHA 2	H-L^{158}PKKAAPAKKAAPAKKAA-NH_2
HBHA2 M	$(Me)_2$-L^{158}P*KK*AAPA*KK*AAPA*KK*AA-NH_2
HBHA 3	H-L^{158}PKKAAPAKKAA-NH_2
HBHA 3 M	$(Me)_2$-L^{158}P*KK*AAPA*KK*AA-NH_2
HBHA 4	Ac-K^{160}AAPAKKAAPAKKAAPAK-NH_2
HBHA4 M	Ac-*K*^{160}AAPA*KK*AAPA*KK*AAPA*K*-NH_2
nHBHA	Native HBHA protein (methylated in the C-terminal 158–198 domain)
FLAG	H-SDYKDHDGDYKDHDIDYKDDDDKGGS-NH_2
HA	H-SGYPYDVPDYAGYPYDVPDYAGYPYDVPDYAS-NH_2
myc	H-SEQKLISEEDLNGEQKLISEEDLNAEQKLISEEDLG-NH_2

$K(Me)_2$ is symbolized by *K*

peptides HBHA 1–4M (19–21). This procedure converted all the primary amino groups present in the peptide into *N*,*N*-dimethylamino groups (ε-amino groups of Lys residues and α-amino group of the N-terminal amino acid when not acetylated, see Note 6). Tag peptides HA (22), FLAG (23), and *myc* (24), protein A (25), and nHBHA were also incorporated in the microarray.

The probes were printed on amine-functionalized microscope glass slides (see Note 7). Peptide microarrays were reductively methylated as illustrated in Fig. 1 using the same combination of reagents as for soluble peptides (see Note 8). Unmodified parent microarrays and daughter methylated microarrays were incubated with sera from nHBHA-immunized mice, sera from naive mice (adjuvant), or murine anti-HA, anti-FLAG® M2, or anti-*myc* monoclonal antibodies. Detection of captured antibodies was performed using tetramethylrhodamine-labeled anti-murine goat secondary antibodies and a standard fluorescence scanner.

The data presented in Fig. 2a correspond to the parent microarrays incubated with sera from nHBHA-immunized mice. Strong signal strengths were observed for methylated peptides HBHA 1–4M and for nHBHA. Spot intensities for the control peptides HA, FLAG, and *myc* corresponded to the background of the microarray, whereas incubation with the anti-HA, FLAG, or *myc* monoclonal antibodies resulted in strong spot intensities for

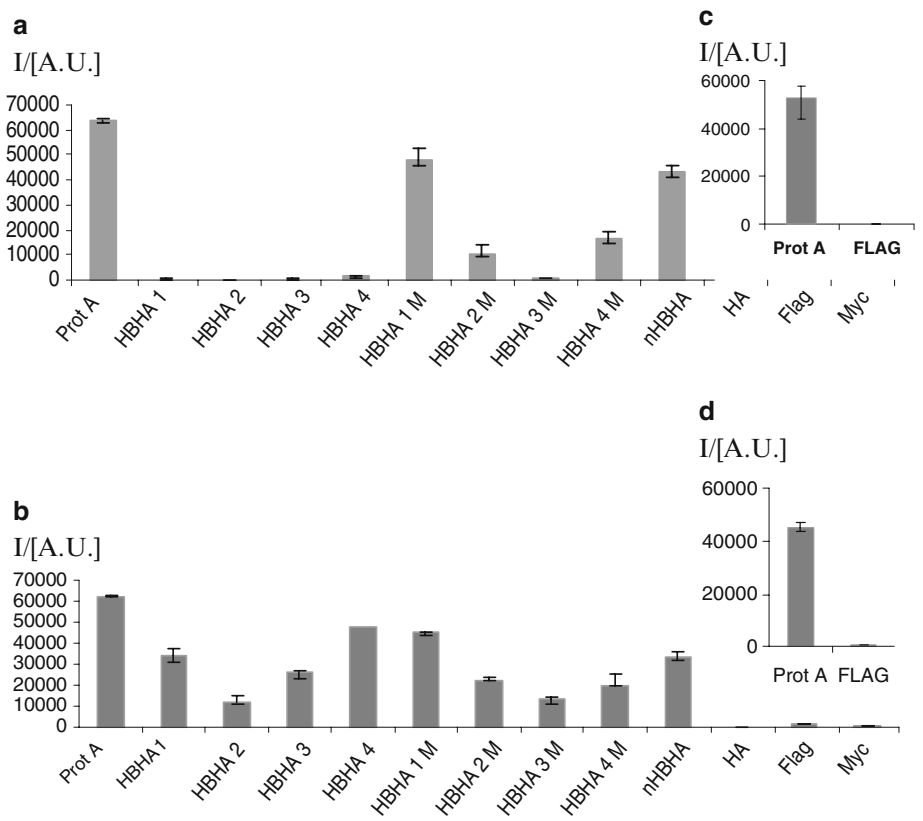


Fig. 2. Quantification of the in situ methylation experiment. Sera were diluted 1/20 in PBS-B. (**a**) Parent microarray and pool of sera from nHBHA-immunized mice, (**b**) daughter (in situ methylated) microarray and pool of sera from nHBHA-immunized mice, (**c**) parent microarray and pool of sera from naïve mice, (**d**) daughter microarray and pool of sera from naïve mice. The signal displayed by the other probes was not significantly different from the background of the microarray (data not shown). The data correspond to the median and interquartile ($n = 3$). Detection was performed at 532 nm.

their cognate antigens (data not shown). Only protein A displayed strong spot intensities after incubating with sera from naive mice (Fig. 2c). These data suggest that anti-nHBHA antibodies directed against the sequence 158–181 of nHBHA recognize preferentially methylated epitopes. The same experiments were performed after in situ methylation (Fig. 2b). In these conditions, strong signal strengths were observed for HBHA 1–4, HBHA 1–4M, and nHBHA but not for the control peptides. These results confirm the presence of the peptides on the substrate and the accessibility of Lys residues within unmodified HBHA 1–4 peptide microspots, and consequently the specificity of antibodies for methylated epitopes. This experiment illustrates also the possibility to generate methylated epitopes in a microarray format by in situ methylation of unmodified peptide probes. The level of methylation can be controlled by varying the concentration of the reagents and the duration of in situ methylation reaction (see accompanying chapter 10).

3.1. Preparation of Amine-Functionalized Microscope Glass Slides

1. Place the microscope glass slides in the glass slide rack. Place the slide rack in the Teflon staining dish with a magnetic bar.
2. In the Teflon® pot add carefully within 1 min 250 mL of concentrated sulfuric acid to 250 mL of hydrogen peroxide under stirring. The reaction is highly exothermic.
3. Once prepared add carefully this solution into the staining dish. The solution must cover the slides.
4. Stir for 1 h and then remove the slide rack from the staining dish; discard the piranha solution in a dedicated waste container (see Note 9).
5. Wash the slide rack with 500 mL of deionized water three times for 3 min, by immersing it in a glass staining dish.
6. Wash similarly with 500 mL of MeOH.
7. Mix 15 mL of 3-aminopropyltrimethoxysilane and 460 mL of MeOH. Add 24.5 mL of deionized water to this solution.
8. Immerse the slide rack in this solution in a glass staining dish.
9. Place the staining dish in a sonicator for 30 min.
10. In the staining dish, wash the slide rack with 500 mL of MeOH, twice with 500 mL of deionized water and finally with 500 mL of methanol.
11. Place the slide rack in the oven at 110°C for 15 min.
12. Remove the slide rack from the oven and place it in the dessicator under vacuum overnight.
13. Control the surface properties of the slides (one slide per reference liquid) (see Note 10).
14. Store the slides at 4°C in a closed box.

3.2. Printing

1. Prepare 20 µL of each peptide or protein solution in a V-bottomed 384-well microtiter plate. The peptides were 0.1 mM in PBS. nHBHA protein was 160 µg/mL in PBS.
2. Print the mixtures (three drops, 300 pL each, three replicates per probe) on amine-functionalized glass slides.
3. Place the microarrays in a closed box at 4°C until use.

3.3. In Situ Methylation (See Notes 11 and 12)

1. Deposit 150 µL of solution 3 on each microarray.
2. Place the cover glass on the slide. The liquid must cover all the microarray.
3. After 2 min at 22°C, remove the cover glass, and rinse the slide copiously with deionized water.
4. Dry the microarray under a stream of nitrogen.
5. Use the methylated microarray immediately in the next step.

3.4. Incubations

1. Use three slides per primary antibody. Deposit 150 µL of PBS-B per slide, place the cover glasses, and incubate for 5 min in the oven (37°C, 60% relative humidity).
2. In the meantime, dilute primary antibody in PBS-B at the appropriate dilution (typically sera were diluted 1/20).
3. Remove the cover glasses and wash the microarrays copiously with PBS-A.
4. Deposit 150 µL of diluted primary antibody on the microarray and place the cover glass. Incubate for 1 h in the oven.
5. In the meantime, dilute secondary antibody in PBS-B (final concentration 10 µg/mL).
6. Remove the cover glasses and wash the microarrays copiously with PBS-A.
7. Deposit 150 µL of diluted secondary antibody on the microarray and place the cover glasses. Incubate for 1 h in the oven.
8. Remove the cover glasses and wash copiously with PBS-A, water, and ethanol.
9. Dry the slides under a stream of nitrogen.
10. Read the slides with the microarray scanner at 532 nm.

4. Notes

1. The piranha solution (H_2SO_4/H_2O_2) is a strong oxidant. It reacts violently with organic materials. It can cause severe skin burns. It must be handled with extreme care in a well-ventilated fume hood while wearing appropriate chemical safety protection.
2. Deionized water has a resistivity of 18 MΩcm.
3. The printing was performed using a noncontact piezoelectric microarrayer (BCA1, Perkin Elmer, MA, USA). Typically, three drops (300 pL each) were printed per spot. Other printing machines can be used as well. For example, we have obtained excellent results using a Qarray2 contact microarrayer (Genetix, New Milton, UK).
4. These solutions must be prepared just before the in situ methylation, especially solution 3.
5. This region is responsible for the binding of the protein to heparan sulfate proteoglycans. The methylation of this functionally important domain protects it against proteolytic degradation (17). Lysines 161, 166, 167, 172, 173, and 178 are mainly dimethylated in nHBHA. These lysines are also dimethylated in HBHA 1–4M.

6. The presence of Ni(II) in this reductive methylation process is essential to avoid the formation of unwanted *N*-cyanomethyl groups, which are formed by nucleophilic addition of cyanide ions on intermediate methylene imine groups. MALDI-TOF spectra of HBHA 1–4M showed only one molecular ion in each case, corresponding to the permethylated peptide.
7. Physisorption of molecules on amine-functionalized glass slides is a simple and efficient method for preparing microarrays (13, 14, 26–29). We have observed as others that amine-functionalized silicon oxide substrates prepared by silanization with 3-aminopropyltrimethoxysilane are rapidly contaminated by air impurities. This contamination leads to a significant increase of water contact angles in only few days (13). Thus, we recommend printing the slides rapidly after substrate preparation. Similarly, it is recommended to store the microarrays at 4°C before use for minimizing both pollution and degradation phenomena.

   Immobilization by physisorption is the method of choice here because the chemical modification of the peptide probes on the microarray can be characterized by mass spectrometry using the desorption ionization on silicon (DIOS) technique (see the accompanying Chapter 10).
8. Side products due to cyanomethylation were not detected when methylated microarrays were analyzed using the DIOS technique (see Note 6 and accompanying Chapter 10).
9. The piranha solution must not be in contact with organic solvents or other aqueous solutions (see also Note 1).
10. Contact angle measurements were performed at 20°C with water, formamide, and diiodomethane as reference liquids. At least nine drops of 1 μL are used for the determination of the contact angles for each liquid. Typical contact angles are $33.1 \pm 0.5°$ for water, $31.5 \pm 0.6°$ for diiodomethane, and $21.5 \pm 0.7°$ for formamide (13).
11. It is also interesting to note that primary amino groups within the 3-aminopropylsilane layer on the microscope glass slide can be potentially methylated too. This modification has apparently no effect on the background of the slide or on the accessibility of the probes.
12. The in situ methylation can also be performed using a microplate microarray hardware 96 wells (4 × 24) (ref. MMH4 × 24) equipped with a silicone gasket (ref. GMMH4 × 24, TeleChem International, Inc., CA, USA). This microarray hardware can accept four slides and each slide can accept 24 microarray patterns. This hardware allows combining the microarray and 96-well formats, the use of standard incubator/agitator for 96-well microtiter plates

(e.g., ref. PST-60HL-4, Biosan, MI, USA) and of standard 96-well microtiter plate washing station (wellwash AC, Thermo Electron, Finland). The in situ methylation was performed using 50 µL of solution 3 per well.

Acknowledgments

We gratefully acknowledge the financial support from CNRS, Université de Lille Nord de France, Institut Pasteur de Lille, IFR 142, Région Nord Pas de Calais, the European Community (FEDER), and from Cancéropôle Nord-Ouest. This research was performed using the Chemistry Systems Biology platform (http://csb.ibl.fr).

References

1. Glokler, J., and Angenendt, P. (2003) Protein and antibody microarray technology. *J Chromatogr B Analyt Technol Biomed Life Sci* **797**, 229–40.
2. Graham, K. L., Robinson, W. H., Steinman, L., and Utz, P. J. (2004) High-throughput methods for measuring autoantibodies in systemic lupus erythematosus and other autoimmune diseases. *Autoimmunity* **37**, 269–72.
3. Hueber, W., Kidd, B. A., Tomooka, B. H., Lee, B. J., Bruce, B., Fries, J. F., Sonderstrup, G., Monach, P., Drijfhout, J. W., van Venrooij, W. J., Utz, P. J., Genovese, M. C., and Robinson, W. H. (2005) Antigen microarray profiling of autoantibodies in rheumatoid arthritis. *Arthritis Rheum* **52**, 2645–55.
4. Neuman de Vegvar, H. E., Amara, R. R., Steinman, L., Utz, P. J., Robinson, H. L., and Robinson, W. H. (2003) Microarray profiling of antibody responses against simian-human immunodeficiency virus: Postchallenge convergence of reactivities independent of host histocompatibility type and vaccine regimen. *J Virol* **77**, 11125–38.
5. Robinson, W. H., DiGennaro, C., Hueber, W., Haab, B. B., Kamachi, M., Dean, E. J., Fournel, S., Fong, D., Genovese, M. C., de Vegvar, H. E., Skriner, K., Hirschberg, D. L., Morris, R. I., Muller, S., Pruijn, G. J., van Venrooij, W. J., Smolen, J. S., Brown, P. O., Steinman, L., and Utz, P. J. (2002) Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat Med* **8**, 295–301.
6. Robinson, W. H., Fontoura, P., Lee, B. J., de Vegvar, H. E., Tom, J., Pedotti, R., DiGennaro, C. D., Mitchell, D. J., Fong, D., Ho, P. P., Ruiz, P. J., Maverakis, E., Stevens, D. B., Bernard, C. C., Martin, R., Kuchroo, V. K., van Noort, J. M., Genain, C. P., Amor, S., Olsson, T., Utz, P. J., Garren, H., and Steinman, L. (2003) Protein microarrays guide tolerizing DNA vaccine treatment of autoimmune encephalomyelitis. *Nat Biotechnol* **21**, 1033–9.
7. Robinson, W. H., Steinman, L., and Utz, P. J. (2002) Protein and peptide array analysis of autoimmune disease. *Biotechniques* **Suppl**, 66–9.
8. Robinson, W. H., Steinman, L., and Utz, P. J. (2003) Protein arrays for autoantibody profiling and fine-specificity mapping. *Proteomics* **3**, 2077–84.
9. Templin, M. F., Stoll, D., Schrenk, M., Traub, P. C., Vohringer, C. F., and Joos, T. O. (2002) Protein microarray technology. *Drug Discov Today* **7**, 815–22.
10. Talapatra, A., Rouse, R., and Hardiman, G. (2002) Protein microarrays: Challenges and promises. *Pharmacogenomics* **3**, 527–36.
11. Ryan, T. E., and Patterson, S. D. (2002) Proteomics: Drug target discovery on an industrial scale. *Trends Biotechnol* **20**, S45–51.
12. Duburcq, X., Olivier, C., Malingue, F., Desmet, R., Bouzidi, A., Zhou, F., Auriault, C., Gras-Masse, H., and Melnyk, O. (2004)

Peptide–protein microarrays for the simultaneous detection of pathogen infections. *Bioconjug Chem* **15**, 307–16.

13. Duburcq, X., Olivier, C., Desmet, R., Halasa, M., Carion, O., Grandidier, B., Heim, T., Stievenard, D., Auriault, C., and Melnyk, O. (2004) Polypeptide semicarbazide glass slide microarrays: Characterization and comparison with amine slides in serodetection studies. *Bioconjug Chem* **15**, 317–25.
14. Mezzasoma, L., Bacarese-Hamilton, T., Di Cristina, M., Rossi, R., Bistoni, F., and Crisanti, A. (2002) Antigen microarrays for serodiagnosis of infectious diseases. *Clin Chem* **48**, 121–30.
15. Petrik, J. (2001) Microarray technology: The future of blood testing? *Vox Sang* **80**, 1–11.
16. Locht, C., Raze, D., Rouanet, C., Genisset, C., Segers, J., and Mascart, F. (2008) The mycobacterial heparin-binding hemagglutinin, virulence factor and antigen useful for diagnostics and vaccine development. In *The Mycobacterial Cell Wall*, edited by N. Daffé and J.-M. Reyrat, ASM Press, Washington DC, 305–322.
17. Pethe, K., Bifani, P., Drobecq, H., Sergheraert, C., Debrie, A. S., Locht, C., and Menozzi, F. D. (2002) Mycobacterial heparin-binding hemagglutinin and laminin-binding protein share antigenic methyllysines that confer resistance to proteolysis. *Proc Natl Acad Sci U S A* **99**, 10759–64.
18. Temmerman, S., Pethe, K., Parra, M., Alonso, S., Rouanet, C., Pickett, T., Drowart, A., Debrie, A. S., Delogu, G., Menozzi, F. D., Sergheraert, C., Brennan, M. J., Mascart, F., and Locht, C. (2004) Methylation-dependent T cell immunity to *Mycobacterium tuberculosis* heparin-binding hemagglutinin. *Nat Med* **10**, 935–41.
19. Gidley, M. J., and Sanders, J. K. (1982) Reductive methylation of proteins with sodium cyanoborohydride. Identification, suppression and possible uses of N-cyanomethyl by-products. *Biochem J* **203**, 331–4.
20. Jentoft, N., and Dearborn, D. G. (1979) Labeling of proteins by reductive methylation using sodium cyanoborohydride. *J Biol Chem* **254**, 4359–65.
21. Means, G. E., and Feeney, R. E. (1990) Chemical modifications of proteins: History and applications. *Bioconjug Chem* **1**, 2–12.
22. Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A., and Wigler, M. (1988) Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol Cell Biol* **8**, 2159–65.
23. Brizzard, B. L., Chubet, R. G., and Vizard, D. L. (1994) Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *Biotechniques* **16**, 730–5.
24. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol* **5**, 3610–6.
25. Richman, D. D., Cleveland, P. H., Oxman, M. N., and Johnson, K. M. (1982) The binding of staphylococcal protein A by the sera of different animal species. *J Immunol* **128**, 2300–5.
26. Souplet, V., Desmet, R., and Melnyk, O. (2007) Imaging of protein layers with an optical microscope for the characterization of peptide microarrays. *J Pept Sci* **13**, 451–7.
27. Marcon, L., Melnyk, O., and Stievenard, D. (2008) Current based antibodies detection from human serum enhanced by secondary antibodies labelled with gold nanoparticles immobilized in a nanogap. *Biosens Bioelectron* **23**, 1185–8.
28. Marcon, L., Stievenard, D., and Melnyk, O. (2007) Electrical detection of human immunoglobulins G from human serum using a microbiosensor. *Biosens Bioelectron* **23**, 81–7.
29. Carion, O., Lefebvre, J., Dubreucq, G., Dahri-Correia, L., Correia, J., and Melnyk, O. (2006) Polysaccharide microarrays for polysaccharide-platelet-derived-growth-factor interaction studies. *Chembiochem* **7**, 817–26.

Chapter 12

Peptide Microarrays on Coated Silicon Slides for Highly Sensitive Antibody Detection

Marina Cretich, Francesco Damin, Renato Longhi, Cecilia Gotti, Clelia Galati, Lucio Renna, and Marcella Chiari

Abstract

Peptides, with their well-established chemistry and fully automated synthesis, provide an invaluable tool for the screening of protein ligands, for epitope mapping, and for antibody diagnostics on the microarray format.

The method described in this chapter shows that the sensitivity of a peptide-based microimmunoassay is greatly improved by using a new, specifically developed substrate made of silicon coated by an optimized layer of silicon oxide. A set of six peptides corresponding to the sequences of human and rat acetylcholine receptor subunits was immobilized on glass and silicon slides coated by a copolymer of *N*,*N*-dimethylacrylamide, *N*-acryloyloxysuccinimide, and 3-(trimethoxysilyl) propyl methacrylate, copoly(DMA–NAS–MAPS). The spotted probes were incubated with rabbit anti-sera and with purified antibodies raised against the corresponding peptides. The coated silicon slides, in comparison against the glass substrates, showed a five- to tenfold enhancement of the fluorescence signals, leading to the specific detection of the full set of antibodies down to a concentration of 0.5–1 ng/mL in serum. The sensitivity provided by the test allows its use for the diagnosis of antibodies in clinical samples.

Key words: Peptides, Microarrays, Silicon, Antibody-based detection, Diagnostics

1. Introduction

Classical methods of protein analysis such as electrophoresis, ELISA, and liquid chromatography are generally time-consuming, labor-intensive, and lack high-throughput capacity (1). Peptide and protein microarrays, with their high-throughput ability and low consumption of reagents, open new opportunities for the study of molecular recognition events in basic biological assays as well as for the development of new diagnostic tools in clinical applications (2–4).

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_12, © Springer Science+Business Media, LLC 2010

Synthetic peptides have some very interesting features as capture ligands in microarray experiments: they are easy to synthesize and manipulate and are usually highly stable and inexpensive. More importantly, since peptide ligands can be modeled to act as a binding site for almost any target structure of the proteome (5), they can mimic biological activities of proteins and provide a straightforward approach in a variety of applications such as the measurement of enzymatic activities (6, 7), the identification of ligands that are active in cell adhesion (8), and the mapping of epitopes (9, 10). Peptide microarrays also provide efficient information in serodiagnosis, for example, in the detection of pathogen infections (11, 12) and for antibody diagnostics (13, 14).

One of the fundamental characteristics of any analytical method is the smallest concentration that can be reliably measured. A high sensitivity is an intrinsically desirable property of any analytical technique as it sets the lower concentrations of analyte that can be distinguished from background noise. A lower assay detection limit allows one to use smaller amounts or more easily obtainable samples, such as capillary blood from newborns or saliva. Moreover, the ability to detect smaller amounts of analyte drastically reduces the influence of the matrix effect (when the combined effect of all the components of sample other than the analyte influences the quantitation) allowing one to dilute the sample. In general, highly sensitive assays open new opportunities in the diagnosis of a disease.

Detection methods developed for microarrays, due to the miniaturized format, provide high sensitivity and high-throughput (15). The use of fluorescent probes and signal amplification techniques with chromogenic or fluorescent labels usually leads to performances that meet such criteria. However, the increased sensitivity in microarray experiments is still a challenge and different approaches to improve detection limits are currently under development. They include alternative labeling involving the use of DNA dendrimers (16), gold (17) or silver (18) nanoparticles, quantum dots (19) or signal amplification methods through the use of tyramide precipitation (20) and the rolling circle amplification (RCA) (21) even combined to nanoparticle-based optical detection (22). These methods provide improvements in sensitivity over the conventional microarray detection technologies, but their applicability is still limited due to the cumbersome procedure required by these methods. The use of novel substrates involving planar waveguides (23) or the phenomenon of fluorescence modulation properties that can be obtained on silicon dioxide substrates as a function of the SiO_2 layer thickness (24) is very promising. The latter approach was used in the development of DNA (25) and protein (26) microarrays. In both works, silicon surface was chemically functionalized based on silanizing agents in order to bind the capture agents.

In this protocol, a specifically developed substrate made of silicon coated by an optimized layer of silicon oxide was

functionalized by a copolymer of *N*,*N*-dimethylacrylamide, *N*-acryloyloxysuccinimide, and 3-(trimethoxysilyl) propyl methacrylate, copoly(DMA–NAS–MAPS) (27, 28). The method proposed for functionalizing silicon dioxide surfaces is simple and robust. It allows exploitation of the fluorescence modulation properties of SiO_2 layer thickness to increase the sensitivity of any microarray analysis. The polymer coating covalently binds peptides in a random conformation reacting with nucleophilic groups in peptidic sequences: amino, thiol, and hydroxyl groups.

A set of six peptides, corresponding to sequences from human and rat brain nicotinic acetylcholine receptors (nAChrs) subunits (see Table 1), was used for the detection of antibodies in rabbit serum in a sandwich immunoassay. Each of the peptides was used to raise specific antibodies in rabbits. The six sera from immunized rabbits were screened with the peptide microarray for the detection of antibodies. The peptides were also used to purify from the rabbit sera, by immunochromatography, specific antibodies that were spiked, at known concentrations, into a diluted aspecific serum. The limits of detection of the sandwich immunoassay on glass were compared to those achieved on silicon slides using the set of reagents reported in Table 1 (peptides, corresponding antisera, and purified antibodies) in a model immunoassay experiment. The optimized silicon dioxide layer on the silicon slides leads to an increase in fluorescence signal of five- to tenfold, in comparison with a common glass slide, allowing the detection of antibodies in serum down to the 0.5–1 ng/mL concentration range which is the level of sensitivity required for the diagnosis of antibodies in clinical samples (14).

Table 1
Details of peptides and antibodies used in this work

Peptide code	Origin	Sequence (N→C)	Antiserum code	Antibody code
A3H	Human, α3 subunit	TRPTSNEGNAQKPRPLYGAELSNLNC	S746	746
A3R	Rat, α3 subunit	CQPLMARDDT	S790	790
A4H	Human, α4 subunit	SPSDQLPPQQPLEAEKASPHPSPGPC	S801	801
A5R	Rat, α5 subunit	CGPVHIGNTIK	S783	783
B4H	Human, β4 subunit	GPDSSPARAFPPSKSCVTKPEATATSPPYG	S808	808
B4R	Rat, β4 subunit	CGPALKMWIHRFH	S758	758

Peptides correspond to sequences from human and rat brain nicotinic acetylcholine receptors (nAChrs) subunits. Rabbit antisera were raised against the corresponding peptide as described in Subheading 3.4. Polyclonal antibodies were purified from the corresponding antisera by immunochromatography as described in Subheading 3.4

2. Materials

2.1. Synthesis of the Copoly(DMA–NAS–MAPS)

1. *N,N*-Dimethylacrylamide (DMA) (Sigma, St. Louis, MO, USA).
2. 3-(Trimethoxysilyl) propyl methacrylate (MAPS) (Sigma, St. Louis, MO, USA).
3. *N*-Acryloyloxysuccinimide (NAS) (Polysciences, Warrington, PA, USA).
4. Tetrahydrofuran (THF) (Sigma, St. Louis, MO, USA).
5. α,α′-Azoisobutyronitrile (AIBN) (Sigma, St. Louis, MO, USA).
6. Petroleum ether (Sigma, St. Louis, MO, USA).

2.2. Derivatization of Glass and Silicon Slides with Copoly(DMA–NAS–MAPS)

1. Untreated glass microscope slides (25×75 mm) (Sigma, St. Louis, MO, USA).
2. Silicon slides (25×75 mm) (ST Microelectronics, Catania, Italy).
3. Pretreatment solutions: ethanol 96% v/v; sodium hydroxide (NaOH) 1 M; hydrogen chloride (HCl) 1 M.
4. Copoly(DMA–NAS–MAPS) synthesized as described in Subheading 3.1.
5. Coating solution: 1% w/v of copoly(DMA–NAS–MAPS) in an aqueous solution of 20% ammonium sulfate.

2.3. Preparation and Spotting of Peptides

1. Lyophilized peptides synthesized by the solid-phase Fmoc method (29) using an Applied Biosystem model 433A Peptide Synthesizer (Foster City, CA).
2. Rabbit IgG and anti-rabbit IgG Cy3 labeled developed in goat (Jackson Immuno Research, USA).
3. Spotting buffer: phosphate-buffered saline solution (PBS 1×) (pH 7.5): 0.137 M NaCl, 2.7 mM KCl, 12 mM Na_2HPO_4, 1.76 mM KH_2PO_4 filtered by a 0.22 μm filter.
4. Genetix QArray2 Microarray Robots (Genetix, Hampshire, UK) equipped with one split spotting pin (Telechem, Sunnyvale, CA, USA).

2.4. Blocking and Incubation of Microarrays

1. Blocking solution: BSA 2% w/v in PBS.
2. Serum samples containing polyclonal antibodies from immunized rabbits were raised against the corresponding peptide and purified according to (30).
3. Anti-rabbit IgG Cy3 labeled developed in goat (Jackson Immuno Research, USA).
4. Incubation buffer: Tris–HCl 0.1 M pH 8, 0.1 M NaCl, 1% w/v BSA, 0.02% w/v Tween 20.

5. Washing buffer: 0.05 M Tris–HCl pH 9, 0.25 M NaCl, 0.05% Tween 20.

2.5. Fluorescent Scanning and Data Analysis

1. ScanArray™ Express (Perkin Elmer, MA, USA), data intensities were extracted with the software associated to the scanner.

3. Methods

The methods described in this section outline (a) the synthesis of the copoly(DMA–NAS–MAPS), (b) the coating of glass and silicon microscope slides with the copoly(DMA–NAS–MAPS), (c) the peptide synthesis, (d) the preparation of antibodies and anti-sera, (e) the spotting and postprocessing of the microarrays, and (f) the microarray experiments.

3.1. Synthesis of the Copoly(DMA–NAS–MAPS)

The copolymer made of DMA (with 97% of moles in the monomer feed), NAS (2% molar percentage), and 3-(trimethoxysilyl) propyl methacrylate (MAPS, 1% molar percentage) is synthesized by free radical copolymerization and used as a functional coating for the fabrication of the peptide microarrays.

1. Dissolve 4 g of DMA, 140.6 mg of NAS, and 13 mg of the initiator azoisobutyronitrile (AIBN) in 42 mL of dried tetrahydrofuran (THF) in a 100 mL-round-bottomed flask, equipped with condenser, magnetic stirring, and nitrogen connection. The concentration of the monomer feed in the solvent is 0.1 g/mL (10% w/v) (see Note 1).
2. Degas the solution by alternating a vacuum and nitrogen purging for 10 min.
3. Dissolve in the same solution 103.3 mg of MAPS.
4. Stir magnetically and warm the solution to 65°C and keep it at this temperature under nitrogen for about 18 h.
5. After the polymerization is completed, precipitate the polymer by pouring the reaction mixture into a large excess of petroleum ether (about 1:10 by volume).
6. Filter the white solid copolymer under vacuum.
7. Dry the copolymer under a vacuum for 1–2 h at room temperature and store it in a dry environment.

3.2. Derivatization of Glass and Silicon Slides with Copoly(DMA–NAS–MAPS)

The coating of glass and silicon slides requires two steps: (a) surface cleaning and pretreatment (different from glass to silicon) and (b) adsorption of the copoly(DMA–NAS–MAPS).

3.2.1. Surface Cleaning and Pretreatment of Glass Slides

1. Clean the microscope glass slides by immersion in pure ethanol for 30 min in a glass chamber at room temperature (see Note 2).
2. Dry the slides for few minutes leaving on the bench to air dry.
3. Incubate slides in a solution of 1 M NaOH for 30 min at room temperature in a glass chamber.
4. Wash slides vigorously two or three times by dipping them, a slide at a time, into a Becker plenty of ddH_2O to remove all traces of NaOH.
5. Dry the slides for few minutes leaving on the bench to air dry.
6. Incubate slides in a solution of 1 N HCl for 1 h at room temperature in a glass chamber.
7. Repeat step 4.
8. Spin dry in a centrifuge at $100 \times g$ for 3 min.

3.2.2. Surface Cleaning and Pretreatment of Silicon Slides

1. Incubate slides in a solution of 0.1 M NaOH for 15 min at room temperature in a glass chamber.
2. Wash slides vigorously two or three times by dipping them, a slide at a time, into a Becker plenty of ddH_2O to remove all traces of NaOH.
3. Spin dry in a centrifuge at $100 \times g$ for 3 min.

3.2.3. Adsorption of the Copoly(DMA–NAS–MAPS) to Glass and Silicon Slides

1. Prepare a coating solution of copoly(DMA–NAS–MAPS), 1% w/v in an aqueous solution of 20% ammonium sulfate (see Note 3).
2. Incubate the glass and silicon slides in the coating solution for 30 min at room temperature in a plastic chamber (see Note 2).
3. Put the slides in a rack and wash them vigorously with ddH_2O to remove the excess of the copolymer on the slides.
4. Dry the slides in a vacuum oven at 80°C for 15 min.
5. Store slides at room temperature in a dessicator, and use slides within 4 weeks after production (see Note 4).

3.3. Peptide Synthesis

The peptides used in this work are reported in Table 1, their sequences correspond to that of human and rat brain nAChrs subunits. Peptides were synthesized by the solid-phase Fmoc method (30) using an Applied Biosystem model 433A Peptide Synthesizer (Foster City, CA). Peptides were lyophilized and then analyzed and purified to apparent homogeneity by reversed-phase high-performance liquid chromatography (RP-HPLC). MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) analysis of the peptides was performed on a Voyager-RP

Biospectrometry Workstation (PE Biosystem, Inc.). Observed experimental values for peptide masses were in agreement with theoretical calculated values.

3.4. Antibodies and Anti-Sera

To obtain antibodies in rabbit, peptides were specifically conjugated to freshly prepared maleimido-activated Mariculture Keyhole Limpet Hemocyanin (mcKLH), using sulfo-SMCC (sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate) (Pierce, Rockford, IL, USA), according to the procedure described by Liu (31). Rabbit polyclonal antibodies were raised against the corresponding peptide and purified according to (30).

3.5. Spotting and Postprint Processing of the Microarray

1. Dissolve the lyophilized peptides, whose details are reported in Table 1, in PBS 1×, at the concentration of 1 mg/mL.
2. Dissolve rabbit IgG and goat Anti-Rabbit IgG in PBS 1× at the concentration of 0.5 mg/mL.

3.5.1. Preparation of the Capture Probes for Spotting

3. Transfer 20 µL of each capture probe in solution into 384-well polypropylene plates.

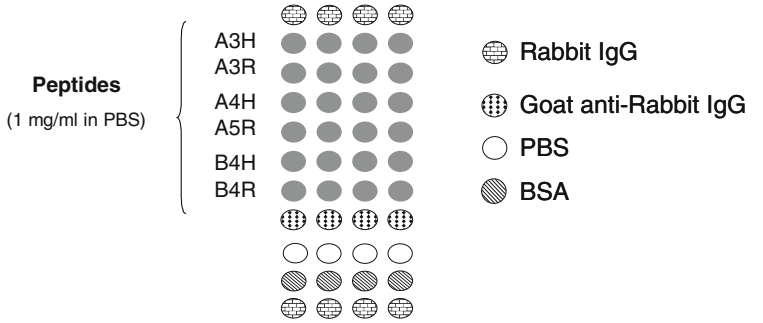
3.5.2. Spotting

The following procedure is optimized onto the Genetix QArray2 Microarray Robots (Genetix, Hampshire, UK). In case other arrayers are used, adjust procedure accordingly.

1. Design a microarray pattern to yield at least four replicates per different capture probe, that is, peptides and positive (rabbit IgG) and negative (PBS and BSA) controls, and to accommodate the probes on every glass and silicon slide according to Scheme 1 (see Note 5).
2. Clean the split spotting pin by sonication 10 min in EtOH and 10 min in H_2O and finally place the pin into the spotting print-head.
3. Set humidity of the robot to about 70% and temperature at 20°C.
4. Program wash routine to clean the pin between different probes. A wash cycle is composed of a wash step with ethanol 80% of 5 s, followed by a wash step with H_2O of 10 s, and a dry step of 15 s with airflow.
5. Incubate the printed silicon and glass slides overnight in a humid chamber at room temperature (see Note 6).

3.5.3. Postprint Processing

1. Rinse all the slides with PBS for 5 min at room temperature and then 5 min with ddH_2O.
2. Incubate all the slides in BSA 1% w/v in PBS at room temperature for 60 min and then rinse with water.



Scheme 1. Spotting scheme. Peptides A3H, A3R, A4H, A5R, B4H, and B4R were spotted at the concentration of 1 mg/mL in PBS. Rabbit IgG and goat anti-rabbit IgG were spotted as positive controls whereas PBS and BSA as negative controls. Proteins were spotted at the concentration of 0.5 mg/mL in PBS. The array was printed with a pitch (distance between the centers of each spot) of 500 μm.

3.6. Microarray Experiments

3.6.1. Sandwich Immunoassay for Antibody Detection in Rabbit Anti-Sera

1. Dilute 1:100 serum samples from immunized rabbits in the incubation buffer (Tris–HCl 0.1 M pH 8, 0.1 M NaCl, 1% w/v BSA, 0.02% w/v Tween 20).
2. Incubate on the printed surface for 1 h at RT.
3. Wash the silicon and glass slides with 0.05 M Tris–HCl pH 9, 0.25 M NaCl, 0.05% Tween 20 for 10 min at room temperature.
4. Rinse with ddH_2O.
5. Dry slides by centrifugation at 100 × *g* for 3 min.
6. Dissolve the secondary Cy3 labeled goat anti-rabbit IgG in the incubation buffer (Tris–HCl 0.1 M pH 8, 0.1 M NaCl, 1% w/v BSA, 0.02% w/v Tween 20) at a concentration of 0.05 mg/mL.
7. Incubate with secondary antibodies at room temperature for 1 h.
8. Wash the slides with PBS at room temperature for 10 min.
9. Wash the slides with ddH_2O at room temperature for 10 min.
10. Dry slides by centrifugation at 100 × *g* for 3 min.

3.6.1.1. Fluorescent Scanning

After binding of the secondary antibody, a fluorescent image of the arrays is acquired by using a confocal laser scanner ScanArray Lite™ (PerkinElmer Life Sciences) equipped with a 550 nm excitation laser.

1. Scan silicon and glass slides at a resolution of 50 μm and adjust laser power and photomultiplier gain to maximize the dynamic range without getting saturation of the signals.
2. Scan slide with a resolution of 10 μm (the results of a typical sandwich assay carried out on one of the six sera, the S801, are shown in Fig. 1).

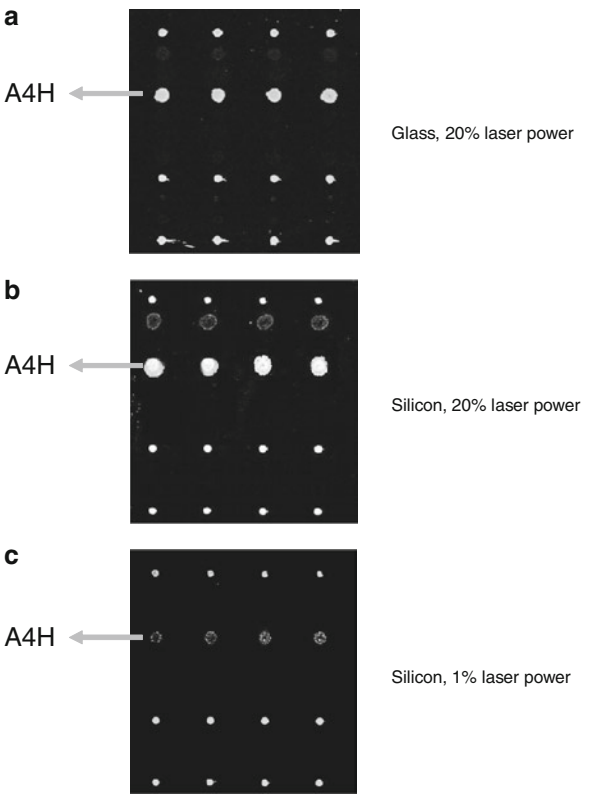


Fig. 1. As an example of the six sandwich immunoassays, results of the screening of serum S801 (that recognizes peptide A4H) are reported. Spots corresponding to peptide A4H are correctly positive, on both substrates silicon and glass slides, due to recognition from specific antibodies in serum S801 (dilution 1:100). (**a**) is the microarray image obtained on copoly(DMA–NAS–MAPS)-coated glass slide, whereas (**b**) is the microarray image obtained on copoly(DMA–NAS–MAPS)-coated silicon slides after scanning the surface using the 20% of the laser power. Fluorescence signals on silicon substrate are higher than signals on glass (five- to tenfold enhancement of the fluorescence signals) leading to saturation of positive control signals. Peptide A4H on silicon is very close to saturation. Therefore, microarray on silicon can be analyzed by lower (1%) laser power without loss of information (**c**).

3.6.2. Peptide Immunoassay Specificity on Coated Silicon Slides

The high specificity of antibodies is crucial for their use in analytical and detection assays. Therefore, specificity and cross-reactivity should be carefully investigated (32). The following competition experiment proves the specificity of the immunoassay carried on the polymer-coated silicon substrate. The peptides in solution competed with those immobilized on the silicon for the binding with the corresponding antibody contained in the serum samples. Capturing of the antibody from the immobilized surface is completely eliminated, leading to a complete absence of the corresponding fluorescence signal.

1. Dilute 1:1000 serum samples from immunized rabbits in the incubation buffer (Tris–HCl 0.1 M pH 8, 0.1 M NaCl, 1% w/v BSA, 0.02% w/v Tween 20).

2. To verify the specificity of the immunoassay, add to the incubation buffer the peptide recognized by the IgG contained in the serum at the concentration of 10 μg/mL.
3. Repeat the steps 2–10 in Subheading 3.6.1.
4. Scan the silicon slides, as described above in Subheading 3.6.1, step 1, to obtain the result shown in the Fig. 2.

3.6.3. Immunoassay Limit of Detection

The sensitivity of a new diagnostic technology determines its success and commercial value (14). Fluorescence-based assays for the detection of proteins, currently, have a sensitivity in the range of pg/L (33); lower detection limits are achievable by radioimmunometric or enzyme-linked assays. Silicon and glass substrates, both coated by copoly(DMA–NAS–MAPS), were tested.

1. Dilute 1:100 aspecific rabbit serum in the incubation buffer (Tris–HCl 0.1 M pH 8, 0.1 M NaCl, 1% w/v BSA, 0.02% w/v Tween 20).
2. Add to the serum each of the six specific immunochromatography purified antibodies (see Table 1) diluted in the 1–0.5 pg/L concentration range.

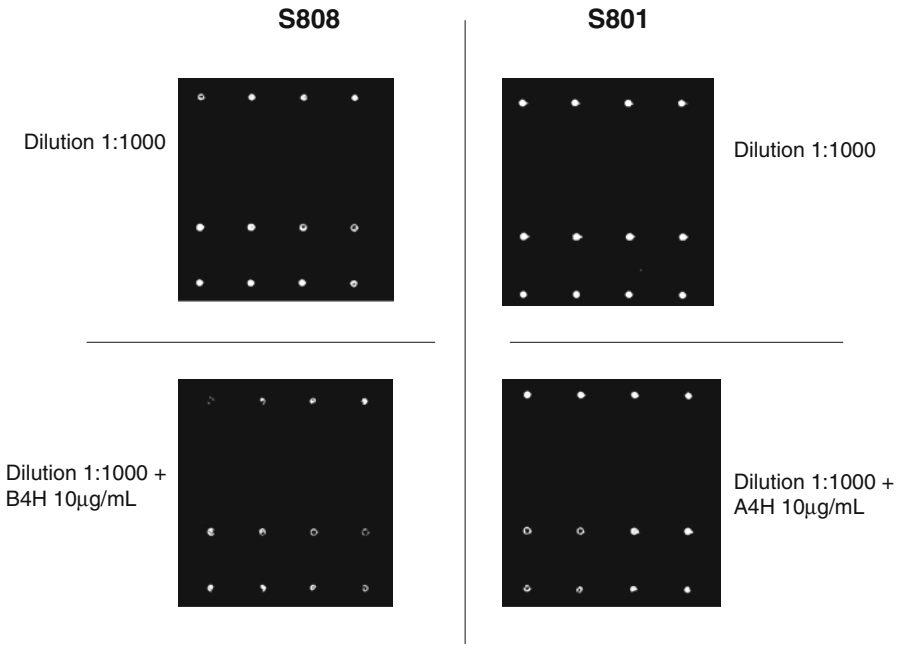


Fig. 2. Assessment of specificity on silicon slides by a competitive experiment. As an example of the six competitive sandwich immunoassays, results of the screening of serum S808 and S801 are shown. In the *upper panels,* peptides B4H and A4H are correctly recognized by specific antibodies contained in serum S808 and S801, respectively. In the *lower panels*, spot capture is completely eliminated due to competition of peptides B4H and A4H spiked in the sera at the concentration of 10 μg/mL. The saturability of the antibody–peptide interaction demonstrates the specificity of the microimmunoassay on copoly(DMA–NAS–MAPS)-coated silicon slides. In this experiment serum samples were diluted 1:1,000 in the incubation buffer. Slides were scanned at 20% laser power.

3. Silicon and glass substrates were subjected to the fluorescence sandwich immunoassay as previously described (see Subheading 3.6.1).

3.6.3.1. Scanning and Evaluation

1. Scan silicon and glass slides at a resolution of 50 μm and adjust laser power and photomultiplier gain to maximize the dynamic range without getting saturation of the signals.
2. Scan slide with a resolution of 10 μm.
3. The fluorescence intensity of the spots was quantified using the software associated to the scanner. The quantification method chosen was the "fixed circle" method. The spot intensity was calculated as the mean intensity of the pixels located within the spot mask minus the mean intensity of the pixels in the background.
4. Data were exported to Microsoft Excel software for further processing and signal intensities were defined in fluorescence arbitrary units.
5. Signal to noise ratios (S/N) for fluorescence signals were calculated as the ratio between the mean spot signal intensity and the standard deviation of background intensity.
6. The limit of detection (LOD) was defined as the lowest concentration of antibody in tested serum at which the mean signal of intensities of the capture spots provided an S/N of 3. The results are reported in Table 2. We extrapolated the

Table 2
Signal to noise ratios (S/N) obtained on copoly(DMA–NAS–MAPS)-coated silicon and glass slides after incubation with purified antibodies spiked in aspecific rabbit serum at the concentrations reported

Antibody code	Concentration (ng/mL)	S/N on silicon	S/N on glass	LOD on silicon (pg/mL)	LOD on glass (pg/mL)
746	0.84	183 ± 48	43 ± 20	14	58
790	0.8	51 ± 18	6.8 ± 3.8	47	353
801	0.7	8.6 ± 3.3	ND[a]	244	ND[b]
783	0.67	9 ± 0.7	ND[a]	223	ND[b]
808	0.52	2 ± 0.6	ND[a]	780	ND[b]
758	1.01	7 ± 2.9	ND[a]	146	ND[b]

In this experiment slides were scanned at 70% laser power, S/N and LOD (limit of detection) were calculated as described in Subheading 3.6.3, step 1. The complete set of the six antibodies, present in the nanogram range in serum, was successfully detected on silicon, whereas only antibodies 746 (0.84 ng/mL) and 790 (0.8 ng/mL) were detected on the glass support

[a]Signal not detected

[b]LOD not determined

limit of detection (LOD), defined as the lowest concentration of antibody in the serum at which the mean signal of intensity provided an S/N of 3, and we obtained, depending on the assayed antibody affinity, a LOD ranging from 780 to 14 pg/mL on silicon and from 353 to 58 pg/mL on glass. Antibodies 801, 783, 808, and 758 at the concentration reported in Table 2 were detected only on the silicon substrate.

4. Notes

1. THF must be distilled under nitrogen, degassed, and stored over dry molecular sieves.
2. It is possible to perform the pretreatment (NaOH and HCl) of the glass slide in glass or plastic chamber indifferently. On the contrary, be sure to use plastic slide chamber for the incubation of the slides in the solution of copoly(DMA–NAS–MAPS) to avoid coating the whole chamber.
3. First, prepare a stock solution of ammonium sulfate at a 40% saturation level. To do this, add 242 g of ammonium sulfate to 1 L of ddH_2O. Depending on the volume of the chamber and the number of the slides to be coated, weigh the exact amount of copolymer to obtain a solution 1% w/v and dissolve it in the half volume of ddH_2O; when the copolymer is completely dissolved, add the remaining volume of ammonium sulfate at a 40%.
4. Glass slides that have been coated with chemical groups are best stored in dry condition in an exsiccator. This is especially important when using the copoly(DMA–NAS–MAPS) because it reacts rapidly with the humidity in the air. The use of copoly(DMA–NAS–MAPS)-coated slides, stored in plastic containers at room temperature for 4 weeks, gave good results.
5. The number of the spots per peptide is a guideline. Remember a rule from statistics: the more events you have, the more accurate the results.
6. Printed slides were placed in an uncovered storage box, laid in a sealed chamber, saturated with sodium chloride, and incubated at room temperature overnight. To make a NaCl-saturated chamber, add as much solid NaCl to water as needed to form a 1-cm-deep slurry in the bottom of a plastic container fitted with an airtight lid. This forms a chamber with a relative humidity of approximately 75%.

References

1. Dupuy, A. M., Lehmann, S., Cristol, J. P. (2005) Protein biochip systems for the clinical laboratory *Clin Chem Lab Med* **43**, 1291–1302.
2. Hu, Y., Uttamchandani, M., Yao, S. Q. (2006) Microarray: a versatile platform for high-throughput functional proteomics *Comb Chem High Throughput Screen* **9**, 203–12.
3. Cretich, M., Damin, F., Pirri, G., Chiari, M. (2006) Protein and peptide arrays: recent trends and new directions *Biomol Eng* **23**, 77–88.
4. Eichler, J. (2005) Synthetic peptide arrays and peptide combinatorial libraries for the exploration of protein-ligand interactions and the design of protein inhibitors *Comb Chem High Throughput Screen* **8**, 135–43.
5. Frank, R. (2002) High-density synthetic peptide microarrays: emerging tools for functional genomics and proteomics *Comb Chem High Throughput Screen* **5**, 429–40.
6. Uttamchandani, M., Chen, G. Y., Lesaicherre, M. L., Yao, S. Q. (2004) Site-specific peptide immobilization strategies for the rapid detection of kinase activity on microarrays *Methods Mol Biol* **264**, 191–204.
7. Cha, T., Guo, A., Zhu, X. Y. (2005) Enzymatic activity on a chip: the critical role of protein orientation *Proteomics* **5**, 416–9.
8. Falsey, J. R., Renil, M., Park, S., Li, S., Lam, K. S. (2001). Peptide and small molecule microarray for high throughput cell adhesion and functional assays *Bioconjug Chem* **12**, 346–53.
9. Chiari, M., Cretich, M., Corti, A., Damin, F., Pirri, G., Longhi, R. (2005) Peptide microarrays for the characterization of antigenic regions of human chromogranin A *Proteomics* **5**, 3600–3.
10. Andresen, H., Zarse, K., Grotzinger, C., Hollidt, J. M., Ehrentreich-Forster, E., Bier, F. F., Kreuzer, O. J (2006) Development of peptide microarrays for epitope mapping of antibodies against the human TSH receptor *J Immunol Methods* **315**, 11–18.
11. Duburcq, X., Olivier, C., Ma lingue, F., Desmet, R., Bouzidi, A., Zhou, F., Auriault, C., Gras-Masse, H., Melnyk, O. (2004) Peptide-protein microarrays for the simultaneous detection of pathogen infections *Bioconjug Chem* **15**, 307–16.
12. Poetz, O., Ostendorp, R., Brocks, B., Schwenk, J. M., Stoll, D., Joos, T. O., Templin, M. F. (2005) Protein microarrays for antibody profiling: specificity and affinity determination on a chip. *Proteomics* **5**, 2402–11.
13. Melnyk, O., Duburcq, X., Olivier, C., Urbes, F., Auriault, C., Gras-Masse, H. (2002) Peptide arrays for highly sensitive and specific antibody-binding fluorescence assays *Bioconjug Chem* **13**,713–20.
14. Andresen, H., Grotzinger, C., Zarse, K., Kreuzer, O. J., Ehrentreich-Forster, E., Bier, F. F. (2006) Functional peptide microarrays for specific and sensitive antibody diagnostics *Proteomics* **6**, 1376–84.
15. Templin, M. F., Stoll, D., Schrenk, M., Traub, P. C., Vohringer, C. F., Joos, T. O. (2002) Protein microarray technology. *Trends Biotechmol* **20**, 160–6.
16. Stears, R. L., Getts, R. C., Gullans, S. R. (2000) A novel, sensitive detection system for high-density microarrays using dendrimer technology *Physiol Genomics* **3**, 93–99.
17. Bao, P., Frutos, A. G., Greef, C., Lahiri, J., Muller, U., Peterson, T. C., Warden, L., Xie, X. (2002) High-sensitivity detection of DNA hybridization on microarrays using resonance light scattering *Anal Chem* **74**, 1792–7.
18. Alexandre, I., Hamels, S., Dufour, S., Collet, J., Zammatteo, N., De Longueville, F., Gala, J. L., Remacle, J (2001) Colorimetric silver detection of DNA microarrays *Anal Biochem* **295**, 1–8.
19. Chan, W. C., Nie, S. (1998) Quantum dot bioconjugates for ultrasensitive nonisotopic detection *Science* **281**, 2016–8.
20. Schweitzer, B., Roberts, S., Grimwade, B., Shao, W., Wang, M., Fu, Q., Shu, Q., Laroche, I., Zhou, Z., Tchernev, V. T., Christiansen, J., Velleca, M., Kingsmore, S. F. (2002) Multiplexed protein profiling on microarrays by rolling-circle amplification *Nat Biotechnol* **20**, 359–65.
21. Schweitzer, B., Wiltshire, S., Lambert, J., O'Malley, S., Kukanskis, K., Zhu, Z., Kingsmore, S. F., Lizardi, P. M., Ward, D. C. (2000) Inaugural article: immunoassays with rolling circle DNA amplification: a versatile platform for ultrasensitive antigen detection *Proc Natl Acad Sci U S A* **97**, 10113–9.
22. Hsu, H. Y., Huang, Y. Y. (2004) RCA combined nanoparticle-based optical detection technique for protein microarray: a novel approach *Biosens Bioelectron* **20**, 123–6.
23. Pawlak, M., Schick, E., Bopp, M. A., Schneider, M. J., Oroszlan, P., Ehrat, M (2002) Zeptosens' protein microarrays: a novel high performance microarray platform for low abundance protein analysis *Proteomics* **2**, 383–93.
24. Lambacher, A., Fromherz, P. (1996) Fluorescence interference-contrast microscopy on oxidized

silicon using a monomolecular dye layer *Appl Phys* **63**, 207–16.

25. Bras, M., Dugas, V., Bessueille, F., Cloarec, J. P., Martin, J. R., Cabrera, M., Chauvet, J. P., Souteyrand, E., Garrigues, M. (2004) Optimisation of a silicon/silicon dioxide substrate for a fluorescence DNA microarray *Biosens Bioelectron* **20**, 797–806.
26. Volle, J. N., Chambon, G., Sayah, A., Reymond, C., Fasel, N., Gijs, M. A. (2003) Enhanced sensitivity detection of protein immobilization by fluorescent interference on oxidized silicon *Biosens Bioelectron* **19**, 457–64.
27. Pirri, G., Damin, F., Chiari, M., Bontempi, E., Depero, L. E. (2004) Characterization of a polymeric adsorbed coating for DNA microarray glass slides *Anal Chem* **76**, 1352–8.
28. Cretich, M., Pirri, G., Damin, F., Solinas, I., Chiari, M. (2004) A new polymeric coating for protein microarrays *Anal Biochem* **332**, 67–74.
29. Fields, G. B., Noble, R. L. (1990) Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids *Int J Peptide Protein Res* **35**, 161–214.
30. Gotti, C., Moretti, M., Bohr, I., Ziabreva, I., Vailati, S., Longhi, R., Riganti, L., Gaimarri, A., McKeith, I. G., Perry, R. H., Aarsland, D., Larsen, J. P., Sher, E., Beattie, R., Clementi, F., Court, J. A. (2006) Selective nicotinic acetylcholine receptor subunit deficits identified in Alzheimer's disease, Parkinson's disease and dementia with Lewy bodies by immunoprecipitation *Neurobiol Dis* **23**, 481–9.
31. Liu, F. T., Zinnecker, M., Hamaoka, T., Katz, D. H. (1979) Procedures for preparation and isolation of protein and Synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates *Biochemistry* **18**, 690–7.
32. Wild, D. (2005) *The immunoassay Handbook*. Elsevier, Oxford, UK.
33. MacBeath, G. (2002) Protein microarrays and proteomics *Nat Genet* **32**, 526–32.

Chapter 13

Antibody Signatures Defined by High-Content Peptide Microarray Analysis

Antonia Masch, Johannes Zerweck, Ulf Reimer, Holger Wenschuh, and Mike Schutkowski

Abstract

Circulating antibodies are highly selective binding reagents directed to a vast repertoire of antigens. Candidate antigens displayed as overlapping peptides on microarrays can be used to screen for recognition by serum antibodies from clinically well-defined patient populations. The methodology is robust and enables unbiased visualization of antigen-specific B-cell responses. Additionally, autoantibody signatures of diagnostic value could be detected using microarrays displaying thousands of human peptides.

Key words: Peptide microarray, Fluorescently labeled antibody, Antibody signatures, Biomarker discovery

1. Introduction

Antigen arrays have been used to gauge humoral immune responses. Here, we improved the resolution down to the epitope level by using high-content peptide microarrays displaying overlapping peptides in the context of infectious and autoimmune diseases, allergy, and cancer.

A detailed knowledge of the target epitopes from pathogens recognized by B-cells either from infected or noninfected (protected) individuals enables rational vaccine design and facilitates the development of novel diagnostic tools. Currently, our knowledge of epitope-specific recognition patterns is limited to small regions of the proteome of pathogens with large genomes. Recently, high-content peptide microarrays have been used to profile immune response to *Mycobacterium tuberculosis* epitopes (1–4) and allowed an unbiased and global view of the immune

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_13,

response, without preselecting target proteins. Peptide microarrays displaying 7,446 unique tuberculosis peptides together with 153 negative controls, 24 positive controls, and 96 fluorescent landmarks were treated with sera from 34 individuals with active pulmonary tuberculosis (TB) and 35 healthy individuals in order to extract significant differences and antibody signatures predictive of tuberculosis. Linear 15mer tuberculosis peptides overlapping by three amino acid residues were printed on functionalized glass slides via a linker on the N-terminus of the peptides. Resulting peptide microarrays can be stored at 4°C for up to 18 months. Sera from patients with active pulmonary tuberculosis were diluted 1:100 using a buffer consisting of PBS, 3% fetal calf serum, and 0.5% Tween and placed onto the microarray surface, incubated at 4°C for 16 h, washed, dried and IgA/IgG-peptide complexes were visualized using fluorescently labeled secondary antihuman-IgA or IgG reagents, respectively.

Three distinct patterns of antibody reactivity were identified: (a) peptides which were differentially recognized between TB + patients and healthy individuals, (b) peptides which were exclusively recognized in all patients with TB but not in any of the healthy individuals, and (c) peptides which were exclusively recognized in healthy individuals but not in TB + patients. Antibody signatures in serum from TB + patients from Armenia versus patients recruited in Sweden showed that IgG-defined tuberculosis epitopes are very similar in individuals with different genetic background. Interestingly, segregation between tuberculosis patients and healthy individuals does not cluster into specific recognition of distinct *M. tuberculosis* proteins, but into specific peptide epitope "hotspots" at different locations within the same protein.

The use of peptide microarrays is not limited to the detection of antibody signatures directed against foreign antigens in infectious diseases (5–10) and allergies (11–15). Peptide microarrays displaying citrullinated peptide derivatives derived from human proteins enabled the detection of induction of antigen-specific tolerance in multiple sclerosis after immunization with DNA encoding myelin basic protein in a randomized, placebo-controlled clinical trial (16, 17). Diluted cerebral spinal fluid was used to probe autoantibody binding to microarray-bound peptides, and it could be demonstrated that the DNA vaccine was able to cross-tolerate to other antigenic components such as proteolipid protein. Moreover, it was shown that peptide microarrays displaying overlapping peptides derived from autoantigens represent a useful tool to detect diagnostic autoantibody signatures in autoimmune diseases like systemic lupus erythemotosus (18–22), diabetis (23), or rheumatoid arthritis (24–27). Recently, it could be demonstrated that peptide microarrays enable the reliable detection of unexpected antibody reactivities against peptides

unique to autosomal dominant forms of dementia (28) or antibodies which are unique for the clinical and pathological subtypes of multiple sclerosis (29).

Finally, recent studies demonstrate that peptide microarrays can be used for the reliable detection of autoantibodies with low serum titers like antitumor-associated antigen antibodies in cancer patients (30). Figure 1 shows the fluorescence image of a peptide microarray treated with serum from a colon cancer patient. The resulting antibody signatures from a patient cohort together with the signatures from healthy individuals encourage the

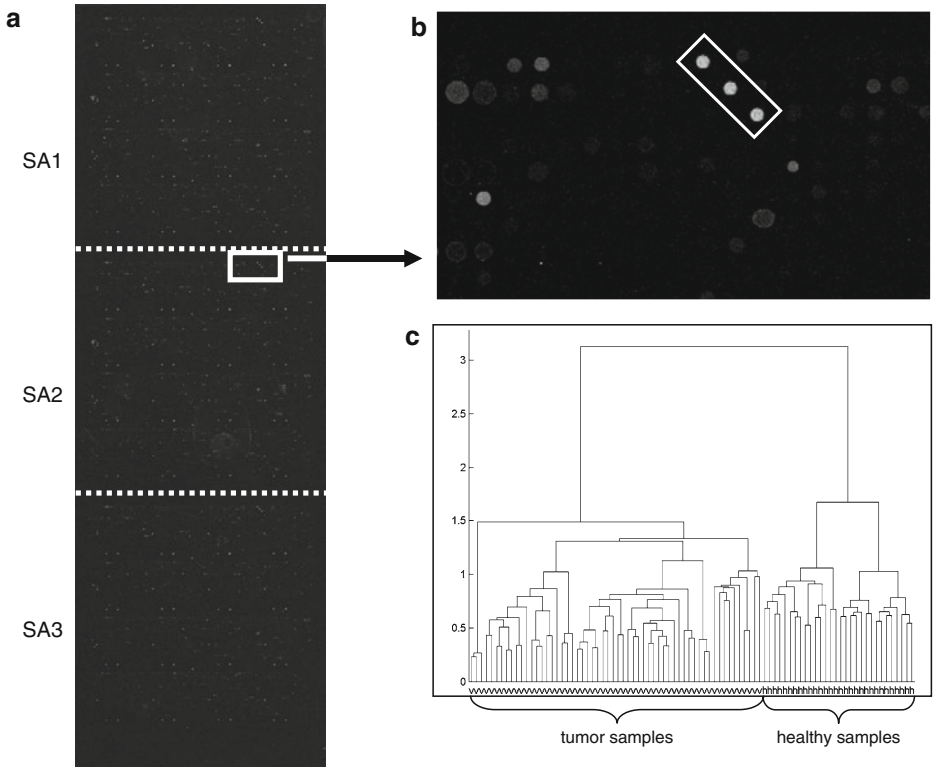


Fig. 1. Overview of peptide microarray analysis. (**a**) Dual color fluorescence image of PepStar microarray consisting of three identical subarrays (SA), each with unique 9,213 spots displaying peptides derived from known tumor-associated antigens plus three fluorescent landmarks used for orientation of the spot recognition software. Peptide microarray was incubated with serum from colon cancer patient (sera were obtained according to the rules and with the approval of the local ethics committees ("Gewebesammlung für molekularbiologische Untersuchungen bösartiger und gutartiger Erkrankungen des Gastrointestinaltraktes und endokriner Organe"; II HV 43/2004) and kindly supplied through Dr. Linnebacher, Section Molecular Oncology and Immune Therapy; University of Rostock, Germany) followed by fluorescently labeled antihuman-IgG antibody. Subsequent to washing and drying steps microarray was scanned with standard microarray reader equipment (GenePix 4000B microarray scanner). (**b**) A magnification shows the three fluorescent landmarks (framed spots) enabling correct orientation of grid generated based on the GenePix array list (.gal-file). Antibody-peptide antigen complexes are visualized using the appropriate fluorescence labeled secondary reagent. White spots represent peptides with bound IgG antibody from patent serum. (**c**) Results of several microarray analyses could be clustered for the determination of diagnostic content.

deduction of content for the development of novel diagnostic/prognostic peptide chips. High-content peptide microarrays represent a robust platform to screen entire proteomes for antibody recognition. This has the potential to define biologically relevant targets in the context of rational vaccine design and the development of novel diagnostics.

2. Materials

2.1. Manual Incubation

2.1.1. Hardware

1. Micropipettes adjustable from 0.5 to 10 µl and from 100 to 1,000 µl with corresponding plastic tips.
2. Petri dishes made of glass.
3. 2 mL Micro Tubes with corresponding Screw caps.
4. Shaking system (Labortechnik Fröbel GmbH, Lindau; Rocky 1000).
5. TaKaRa Spaced Cover Glass XL (Matsunami Glass Ind., Ltd).
6. Microarray High Speed Centrifuge (ArrayIt, MHC220V).
7. Microarray Fluorescence Scanner or Imaging system which is able to perform microarray fluorescence scans with at least 10 µm/pixel resolution (i.e. Axon GenePix 4000B or 4200AL).

2.1.2. Buffers and Reagents

1. TBS-buffer: 50 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, pH 8.0.
2. TBS-based blocking buffer (Superblock T20, #37536, Pierce, Rockford, USA).
3. Fluorescently labeled secondary antibody (mouse antihuman-IgG (H+L) Cy5 conjugated, #209-175-082, Jackson ImmunoResearch Laboratories Inc., USA).
4. Peptide microarrays (JPT Peptide Technologies GmbH, Berlin, Germany) (see Note 1).

2.2. Semiautomatic Incubation

2.2.1. Hardware

1. HS400 microarray processing station, including incubation chambers suitable for industry glass slide format (1 × 3 in.).
2. Gilson Microman Pipetting system (250 µL).
3. Microarray fluorescence scanner or imaging system which is able to perform microarray fluorescence scans with at least 10 µm/pixel resolution.
4. Nitrogen supply connected to HS400 microarray processing station for slide drying.

2.2.2. Buffers and Reagents

1. TBS-buffer plus 0.1% Tween 20: 50 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, pH 8.0.

2. SSC-buffer: 15 mM NaCl, 1.5 mM tri-sodium citrate, pH 7.0.
3. TBS-based blocking buffer (Superblock T20, #37536, Pierce, Rockford, USA).
4. Fluorescently labeled secondary antibody (mouse antihuman-IgG (H+L) Cy5 conjugated, #209-175-082, Jackson ImmunoResearch Laboratories Inc., USA).
5. Peptide microarrays (JPT Peptide Technologies GmbH, Berlin, Germany) (see Note 1).

2.2.3. Software

1. Spot-recognition software like GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA).
2. Microarray evaluation database Acuity 4.0 (Molecular Devices, Sunnyvale, CA).

3. Methods

3.1. Manual Incubation

1. Pretreat the microarrays with blocking buffer. Make sure that the slides are totally submerged in the solution, and shake the slides for 1 h on the orbital shaking system (see Notes 2–10).
2. Wash the slides with TBS-buffer (three times, 1 min each).
3. Wash the slides with double distilled water (three times, 1 min each).
4. Dry the slides using microarray centrifuge.
5. Prepare a humidifying chamber by placing a wet wipe in a vessel which can be tightly sealed and put a Petri dish upside down onto it. Place the microarray slide on top of the Petri dish. By closing the vessel, a constant humidity within the vessel will be formed minimizing evaporation of water from the assay solution.
6. Prepare dilution of the serum in TBS-based blocking buffer. Dilutions to be applied to the microarray ranges from 1:50 to 1:500 (v/v). Using a TaKaRa Spaced Cover Glass the total volume needed is 200 µL.
7. Pipette the diluted serum onto the surface of the microarray (see Note 6). Subsequently, cover the assay solution with the TaKaRa Spaced Cover Glass. Make sure that there are no bubbles within the created incubation chamber. If the cover glass is used correctly, excess solution will be visible at the top and lower end of the cover glass.
8. Seal the humidifying chamber and incubate for 2 h at room temperature.

9. Open the humidifying chamber and remove assay solution as fast as possible by dipping microarrays in large excess of TBS-buffer.
10. Wash the microarray with large volumes of TBS-buffer using shaking system (three times, 5 min each). Make sure that the microarray is totally submerged in the washing solution.
11. Prepare a solution of blocking buffer. Dilute secondary fluorescently labeled antibody with the blocking buffer to a final concentration of 0.1 μg/mL (see Note 11). Submerge the microarray in the solution of secondary antibody using a Petri dish and incubate for 45 min using the shaking system (see Note 12). The total volume for the incubation with the secondary antibody should be about 10 mL. Do not use the TaKaRa Cover Glasses for the secondary antibody but instead incubate the microarray directly in secondary antibody-containing solution.
12. Wash the microarray thoroughly with TBS-buffer (three times, 5 min each) and double distilled water (three times, 5 min each).
13. Rinse front and back of the microarray with double distilled water for 10 s each side.
14. Spin-dry the microarray using a microarray centrifuge.
15. Scan the dried microarray using a microarray scanner with appropriate laser settings (see Notes 13 and 14).
16. Apply GenePix Array List (.gal) files to the scanned images using spot-recognition software. Save the resulting values for the signal intensity and local background.
17. Import the result files to a microarray analysis database for detailed evaluation (see Note 15).

3.2. Semiautomatic Incubation

1. Clip the microarray into the microarray slide holder provided by the HS400 microarray processing station and close the incubation chambers.
2. Make sure that enough liquid is available (2 L of TBS Tween20 (channels 1 and 2, Ch.: 1 and 2)), 1 L of SSC-buffer (channel 5 and 6, Ch.: 5 and 6) and 0.5 L of double distilled water (channel 3, Ch.:3).
3. Prepare the program steps according to the lines shown below (TECAN programming language):
   (a) Step 1: WASH Temp. °C: 30.0, First: Yes, Ch.: 1, Runs: 1, Wash time: 0:02:30, Soak time: 0:00:30
   (b) Step 2: PROBE INJECTION Temp. °C: 30.0
   (c) Step 3: HYBRIDIZATION Temp. °C: 30.0, Agitation Frequency: High, Time: 0:30:00

(d) Step 4: WASH Temp. °C: 30.0, First: No, Ch.: 1, Runs: 3, Wash time: 0:02:30, Soak time: 0:00:30

(e) Step 5: PROBE INJECTION Temp. °C: 30.0

(f) Step 6: HYBRIDIZATION Temp. °C: 30.0, Agitation Frequency: High, Time: 2:00:00

(g) Step 7: WASH Temp. °C: 30.0, First: No, Ch.: 2, Runs: 3, Wash time: 0:02:30, Soak time: 0:00:30

(h) Step 8: PROBE INJECTION Temp. °C: 30.0

(i) Step 9: HYBRIDIZATION Temp. °C: 30.0, Agitation Frequency: High, Time: 0:45:00

(j) Step 10: WASH Temp. °C: 30.0, First: No, Ch.: 2, Runs: 2, Wash time: 0:02:30, Soak time: 0:00:30

(k) Step 11: WASH Temp. °C: 30.0, First: No, Ch.: 5, Runs: 4, Wash time: 0:02:30, Soak time: 0:00:30

(l) Step 12: WASH Temp. °C: 30.0, First: No, Ch.: 6, Runs: 4, Wash time: 0:02:30, Soak time: 0:00:30

(m) Step 13: SLIDE DRYING Temp. °C: 30.0, Time: 0:05:00, Final Manifold Cleaning: Yes, Ch.: 3

(n) Liquid identification:

Liquid for channel 1: TBS 1× 0.1% Tween20

Liquid for channel 2: TBS 1× 0.1% Tween20

Liquid for channel 3: ddwater

Liquid for channel 4: Unused

Liquid for channel 5: SSC 0.1x

Liquid for channel 6: SSC 0.1x

4. Make sure that the nitrogen supply is connected properly to the HS400 station.
5. Start the program and follow the instructions according to the program steps (see Note 16).
6. When finished, open the incubation chambers (see Note 17) and scan the dried microarray using a microarray scanner with appropriate laser settings (see Notes 13 and 14).
7. Apply .gal-files to the scanned images using spot-recognition software. Save the resulting values for the signal intensity and background.
8. Import the result files to a microarray analysis database for detailed evaluation (see Note 15).

3.3. Normalization of Data Sets

3.3.1. Use of Immunoglobulin Control Spots

On each peptide microarray positive controls for the fluorescently labeled secondary antibody are immobilized by crosslinking human full length immunoglobulin via its lysine side chain amino functions. The mean of the corrected signal intensities of all immunoglobulin controls can be used for the normalization of

the data (1) by transforming the corrected signal intensities for the peptide spots into values relative to the mean of the immunoglobulin controls (see Note 18).

3.3.2. Use of False Positives

Depending on the nature of the fluorescently labeled secondary antibody and on the peptide sequences deposited onto the microarray, there will be some reactivity against immobilized peptides. These so-called false positives could be used for the normalization of peptide microarray data. Such false positives have to be determined in a control incubation of the respective peptide microarray using buffer followed by the secondary antibody (see Note 15). The mean of the corrected signal intensities of all false positives can be used for the normalization of the data by transforming the corrected signal intensities for the peptide spots into values relative to the mean of the false positives (3).

4. Notes

1. Other peptide microarrays could be used as well. We strongly recommend to use peptide microarrays, where the peptides are immobilized covalently in an oriented manner. Ideally, long and hydrophilic linkers between the peptide and the slide surface like the Ttds-linker introduced by Falsey et al. (31) were successfully used for the definition of antibody signatures (1–3, 8, 18, 32).
2. Always handle peptide microarray slides with care.
3. Never touch the peptide microarray slide surface.
4. Always wear laboratory gloves when handling peptide microarray slides.
5. If a JPT-microarray is used to hold peptide microarray at the end, which carries the engraved lot-and-slide number. If microarrays from other suppliers are used, make sure not to touch the peptides displaying surface.
6. Please take care when dispensing solutions onto the slide surface. Make sure not to touch the surface with pipette-tips or dispensers.
7. Never whisk the surface of the peptide microarray slide with a cloth or similar tools.
8. Never use other chemicals as described. Inappropriate chemicals may destroy the chemical bond between the peptides and the glass surface.

9. Avoid dust or other particles during each step of the experiment. Dust, particles and resulting scratches will cause artifacts during the final signal readout.
10. Filter all solutions for the washing steps through 2 µm, preferably 0.4 µm particle filters before use.
11. Carefully adjust the final dilution of your labeled secondary antibody. Microarray technology is very sensitive, and therefore it could be possible to use the secondary antibody in a higher dilution as proposed by the manufacturer. Generally, 1:1,000 dilutions of a 1 mg/mL stock solution are working very well. Nevertheless, depending on the nature of the secondary antibody, such concentrations may yield high background signals caused by unspecific binding to the coated glass surface. Additionally, number of false positive signals caused by unspecific binding of the secondary reagent to displayed peptides could be too high, if dilution of the secondary reagent is not optimal. In the case of the secondary antibody recommended by us (mouse antihuman-IgG (H + L) Cy5 conjugated, #209-175-082, Jackson ImmunoResearch Laborities Inc, USA), the best working dilution is 1:10,000. If the signals within the peptide spots are high, you could test 1:30,000 dilutions of a 1 mg/mL stock as well.
12. For incubation with the fluorescently labeled secondary antibody, it is important to cover the trays completely with aluminia foil as these antibodies are sensitive toward light.
13. Make sure to scan the front face of the microarray slide carrying the peptides.
14. Fluorescence scanning could be very sensitive depending on the used scanner. Avoid any fluorescent impurities/contaminations inside your assay solution or washing solutions. You can easily check for such impurities by incubating and washing a dummy slide with the same solutions (but not with the fluorescently labeled secondary reagent!) followed by fluorescence imaging.
15. Control incubations using fluorescently labeled secondary antibody should be performed in parallel to the serum experiment to ensure that found signals are not caused by unspecific binding of the secondary antibody. For that experiment, it is important to use a preincubation with the assay buffer but without the diluted serum under same conditions before treatment with the secondary reagent. A direct incubation with the secondary reagent without the preincubation, which is simulating the serum incubation step, could cause false-negative signals. If the number of resulting signals in the control incubation is too high, repeat the experiment with higher dilutions of the secondary reagent.

16. Use excess volumes for all pipetting steps to make sure that the incubation chamber is completely full.
17. Take care when opening and closing the incubation chamber. In case the microarray sticks to the sealing of the chamber, it might be damaged.
18. Please note that in some cases the corrected signal intensity for the peptide spots could be higher than the mean of the immunoglobulin controls resulting in relative values higher than 100%. One reason for that seemingly surprising result is the difference in the immobilization chemistry used. Whereas peptides are immobilized in a directed manner (each individual peptide molecule within the peptide spot is accessible to the secondary antibody), the immunoglobulin controls are immobilized by crosslinking resulting in a mixture of differently orientated individual immunoglobulin molecules within the control spot. Some of the individual immunoglobulin molecules will be immobilized in such a way that the epitope for the fluorescently labeled secondary antibody is not presented properly or hidden.

References

1. Nahtman, T., Jernberg, A., Mahdavifar, S., Zerweck, J., Schutkowski, M., Maeurer, M., and Reilly, M. (2007) Validation of peptide epitope microarray experiments and extraction of quality data, *J Immunol Methods 328*, 1–13.
2. Gaseitsiwe, S., Valentini, D., Mahdavifar, S., Magalhaes, I., Hoft, D. F., Zerweck, J., Schutkowski, M., Andersson, J., Reilly, M., and Maeurer, M. J. (2008) Pattern recognition in pulmonary tuberculosis defined by high content peptide microarray chip analysis representing 61 proteins from *M. tuberculosis*, *PLoS One 3*, e3840.
3. Ngo, Y., Advani, R., Valentini, D., Gaseitsiwe, S., Mahdavifar, S., Maeurer, M., and Reilly, M. (2009) Identification and testing of control peptides for antigen microarrays, *J Immunol Methods 343*, 68–78.
4. Gaseitsiwe, S., Valentini, D., Mahdavifar, S., Reilly, M., Ehrnst, A., and Maeurer, M. (2010) Peptide microarray – based identification of *Mycobacterium tuberculosis* epitope binding to HLA-DRB1*0101, DRB1*1501 and DRB1*0401, *Clin Vaccine Immunol*, 17(1): 168–75.
5. Gaseitsiwe, S., Valentini, D., Ahmed, R., Mahdavifar, S., Magalhaes, I., Zerweck, J., Schutkowski, M., Gautherot, E., Montero, F., Ehrnst, A., Reilly, M., and Maeurer, M. (2009) Major histocompatibility complex class II molecule-human immunodeficiency virus peptide analysis using a microarray chip, *Clin Vaccine Immunol 16*, 567–573.
6. Melnyk, O., Duburcq, X., Olivier, C., Urbes, F., Auriault, C., and Gras-Masse, H. (2002) Peptide arrays for highly sensitive and specific antibody-binding fluorescence assays, *Bioconjug Chem 13*, 713–720.
7. Duburcq, X., Olivier, C., Malingue, F., Desmet, R., Bouzidi, A., Zhou, F., Auriault, C., Gras-Masse, H., and Melnyk, O. (2004) Peptide-protein microarrays for the simultaneous detection of pathogen infections, *Bioconjug Chem 15*, 307–316.
8. Bongartz, J., Bruni, N., and Or-Guil, M. (2009) Epitope mapping using randomly generated peptide libraries, *Methods Mol Biol 524*, 237–246.
9. Neuman de Vegvar, H. E., Amara, R. R., Steinman, L., Utz, P. J., Robinson, H. L., and Robinson, W. H. (2003) Microarray profiling of antibody responses against simian-human immunodeficiency virus: postchallenge convergence of reactivities independent of host histocompatibility type and vaccine regimen, *J Virol 77*, 11125–11138.
10. Neuman de Vegvar, H. E., and Robinson, W. H. (2004) Microarray profiling of antiviral antibodies for the development of diagnostics,

vaccines, and therapeutics, *Clin Immunol 111*, 196–201.

11. Cerecedo, I., Zamora, J., Shreffler, W. G., Lin, J., Bardina, L., Dieguez, M. C., Wang, J., Muriel, A., de la Hoz, B., and Sampson, H. A. (2008) Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay, *J Allergy Clin Immunol 122*, 589–594.
12. Lin, J., Bardina, L., and Shreffler, W. G. (2009) Microarrayed allergen molecules for diagnostics of allergy, *Methods Mol Biol 524*, 259–272.
13. Lin, J., Bardina, L., Shreffler, W. G., Andreae, D. A., Ge, Y., Wang, J., Bruni, F. M., Fu, Z., Han, Y., and Sampson, H. A. (2009) Development of a novel peptide microarray for large-scale epitope mapping of food allergens, *J Allergy Clin Immunol 124*, 315–322, 322. e311–313.
14. Shreffler, W. G., Lencer, D. A., Bardina, L., and Sampson, H. A. (2005) IgE and IgG4 epitope mapping by microarray immunoassay reveals the diversity of immune response to the peanut allergen, Ara h 2, *J Allergy Clin Immunol 116*, 893–899.
15. Flinterman, A. E., Knol, E. F., Lencer, D., Bardina, L., den Hartog Jager, C. F., Lin, J., Pasmans, S. G., Bruijnzeel-Koomen, C. A., Sampson, H. A., van Hoffen, E., and Shreffler, W. G. (2008) Peanut epitopes for IgE and IgG4 in peanut-sensitized children in relation to severity of peanut allergy, *J Allergy Clin Immunol. 121*, 737–743.
16. Bar-Or, A., Vollmer, T., Antel, J., Arnold, D. L., Bodner, C. A., Campagnolo, D., Gianettoni, J., Jalili, F., Kachuck, N., Lapierre, Y., Niino, M., Oger, J., Price, M., Rhodes, S., Robinson, W. H., Shi, F. D., Utz, P. J., Valone, F., Weiner, L., Steinman, L., and Garren, H. (2007) Induction of antigen-specific tolerance in multiple sclerosis after immunization with DNA encoding myelin basic protein in a randomized, placebo-controlled phase 1/2 trial, *Arch Neurol 64*, 1407–1415.
17. Garren, H., Robinson, W. H., Krasulova, E., Havrdova, E., Nadj, C., Selmaj, K., Losy, J., Nadj, I., Radue, E. W., Kidd, B. A., Gianettoni, J., Tersini, K., Utz, P. J., Valone, F., and Steinman, L. (2008) Phase 2 trial of a DNA vaccine encoding myelin basic protein for multiple sclerosis, *Ann Neurol 63*, 611–620.
18. Lorenz, P., Kreutzer, M., Zerweck, J., Schutkowski, M., and Thiesen, H. J. (2009) Probing the epitope signatures of IgG antibodies in human serum from patients with autoimmune disease, *Methods Mol Biol 524*, 247–258.
19. Graham, K. L., Robinson, W. H., Steinman, L., and Utz, P. J. (2004) High-throughput methods for measuring autoantibodies in systemic lupus erythematosus and other autoimmune diseases, *Autoimmunity 37*, 269–272.
20. Robinson, W. H., Steinman, L., and Utz, P. J. (2002) Protein and peptide array analysis of autoimmune disease, *Biotechniques Suppl*, 66–69.
21. Robinson, W. H., Steinman, L., and Utz, P. J. (2003) Protein arrays for autoantibody profiling and fine-specificity mapping, *Proteomics 3*, 2077–2084.
22. Hueber, W., Zeng, D., Sharpe, O., Robinson, W. H., Strober, S., and Utz, P. J. (2004) Characterization of novel antigens recognized by serum autoantibodies from anti-CD1 TCR-transgenic lupus mice, *Eur J Immunol 34*, 1654–1662.
23. Quintana, F. J., Hagedorn, P. H., Elizur, G., Merbl, Y., Domany, E., and Cohen, I. R. (2004) Functional immunomics: microarray analysis of IgG autoantibody repertoires predicts the future response of mice to induced diabetes, *Proc Natl Acad Sci U S A 101 Suppl 2*, 14615–14621.
24. Hueber, W., Kidd, B. A., Tomooka, B. H., Lee, B. J., Bruce, B., Fries, J. F., Sonderstrup, G., Monach, P., Drijfhout, J. W., van Venrooij, W. J., Utz, P. J., Genovese, M. C., and Robinson, W. H. (2005) Antigen microarray profiling of autoantibodies in rheumatoid arthritis, *Arthritis Rheum 52*, 2645–2655.
25. Hueber, W., and Robinson, W. H. (2006) Proteomic biomarkers for autoimmune disease, *Proteomics 6*, 4100–4105.
26. Robinson, W. H., DiGennaro, C., Hueber, W., Haab, B. B., Kamachi, M., Dean, E. J., Fournel, S., Fong, D., Genovese, M. C., de Vegvar, H. E., Skriner, K., Hirschberg, D. L., Morris, R. I., Muller, S., Pruijn, G. J., van Venrooij, W. J., Smolen, J. S., Brown, P. O., Steinman, L., and Utz, P. J. (2002) Autoantigen microarrays for multiplex characterization of autoantibody responses, *Nat Med 8*, 295–301.
27. Kidd, B. A., Ho, P. P., Sharpe, O., Zhao, X., Tomooka, B. H., Kanter, J. L., Steinman, L., and Robinson, W. H. (2008) Epitope spreading to citrullinated antigens in mouse models of autoimmune arthritis and demyelination, *Arthritis Res Ther 10*, R119.
28. Britschgi, M., Olin, C. E., Johns, H. T., Takeda-Uchimura, Y., LeMieux, M. C., Rufibach, K., Rajadas, J., Zhang, H., Tomooka, B., Robinson, W. H., Clark, C. M., Fagan, A. M., Galasko, D. R., Holtzman, D. M., Jutel, M., Kaye, J. A., Lemere, C. A., Leszek, J., Li, G., Peskind, E. R., Quinn, J. F., Yesavage, J. A., Ghiso, J. A.,

and Wyss-Coray, T. (2009) Neuroprotective natural antibodies to assemblies of amyloidogenic peptides decrease with normal aging and advancing Alzheimer's disease, *Proc Natl Acad Sci U S A 106*, 12145–12150.

29. Quintana, F. J., Farez, M. F., Viglietta, V., Iglesias, A. H., Merbl, Y., Izquierdo, G., Lucas, M., Basso, A. S., Khoury, S. J., Lucchinetti, C. F., Cohen, I. R., and Weiner, H. L. (2008) Antigen microarrays identify unique serum autoantibody signatures in clinical and pathologic subtypes of multiple sclerosis, *Proc Natl Acad Sci U S A 105*, 18889–18894.
30. Merbl, Y., Itzchak, R., Vider-Shalit, T., Louzoun, Y., Quintana, F. J., Vadai, E., Eisenbach, L., and Cohen, I. R. (2009) A systems immunology approach to the host-tumor interaction: large-scale patterns of natural autoantibodies distinguish healthy and tumor-bearing mice, *PLoS One 4*, e6053.
31. Falsey, J. R., Renil, M., Park, S., Li, S., and Lam, K. S. (2001) Peptide and small molecule microarray for high throughput cell adhesion and functional assays, *Bioconjug Chem 12*, 346–353.
32. Tapia, V., Bongartz, J., Schutkowski, M., Bruni, N., Weiser, A., Ay, B., Volkmer, R., and Or-Guil, M. (2007) Affinity profiling using the peptide microarray technology: a case study, *Anal Biochem 363*, 108–118.

Chapter 14

High Density Peptide Microarrays for Proteome-Wide Fingerprinting of Kinase Activities in Cell Lysates

Alexandra Thiele, Matthias Weiwad, Johannes Zerweck, Gunter Fischer, and Mike Schutkowski

Abstract

Protein function is highly regulated in pathways that are responsible for complex biochemical mechanisms such as growth, metabolism, and signal transduction. One of the most important mechanisms is posttranslational modification (PTM) changing protein surfaces by phosphorylation, sulfation, acetylation, methylation, glycosylation, and sumoylation resulting in a more than 100-fold higher complexity (Geiss-Friedlander and Melchior, *Nat Rev Mol Cell Biol 8*, 947–956, 2007; Hunter, *Mol Cell 28*, 730–738, 2007). This chapter presents a very efficient way to detect potential phosphorylation sites in protein families using overlapping peptides covering the complete primary structures (peptide scans) immobilized on glass slides.

Results of kinase activity fingerprinting of cell lysates using peptide microarrays displaying peptide scans through all human peptidyl-prolyl-*cis/trans*-isomerases are shown.

Key words: Peptide microarray, Cell lysate, Protein kinase, Tyrosine kinase, Serine/threonine kinase, Phosphorylation sites, Peptidyl-prolyl-*cis/trans*-isomerase, FKBP, Cyclophilin, Pin1, Parvulin, Overlapping peptides, Fluorescent readout, Phosphate-specific dye

1. Introduction

Protein phosphorylation is one of the most important events in signal transduction. There are 518 human genes encoding kinases which are able to modify serines, threonines, and tyrosines resulting in more than 100,000 human phosphorylation sites (3, 4).

The functions of phosphorylation are diverse and can result in either modulation of enzymatic activity by factor of up to 1,000 (5, 6) or it can create binding sites for interacting proteins, and can lead to changes in the localization of the phosphorylated protein by modifying localization sequences (7, 8).

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_14, © Springer Science+Business Media, LLC 2010

The importance of protein phosphorylation becomes obvious in cells with phosphorylation defects as abnormalities can lead to cancer, inflammation, and diabetes.

Most methods to monitor endogenous protein kinase activity in cell lysates depend on the incubation of recombinant protein substrates in the presence of radioisotopically labeled ATP. We replaced the hazardous autoradiography/phosphorimaging by fluorescence imaging using a fluorescently labeled phosphate-specific dye.

As commonly used methods like filter-binding assays or gel-based assays are low in throughput robust methods are needed enabling fast and efficient analysis of kinase modulating processes in cell lysates in response to stimulations by drugs, natural compounds, or second messengers. In contrast to recombinant protein substrates, the peptide substrates printed on glass slides are stable, easy to design, and easier to handle and thus allows the rapid detection of changes in the activities of protein kinases and phosphatases in cell lysates.

Here, we provide a robust peptide microarray protocol to determine phosphorylation sites of complete protein family of interest depending on the activation state of cells. We searched systematically for phosphorylation sites in human peptidyl-prolyl-*cis/trans*-isomerases (PPIases) in a proteome-wide manner as this modification was shown to regulate both the localization of PPIases and the interaction pattern of PPIases with other proteins (8–10). Additionally, phosphorylation is expected to be one mechanism to regulate PPIase enzymatic activity. The PPIases are a family of enzymes catalyzing isomerization of peptidyl-prolyl bonds. This isomerization seems to be important for transport through membranes, cell cycle progression, channel gating, and virus replication (11, 12). Depending on their primary structure and drug-binding ability, PPIases are subdivided into three distinct families: cyclophilins (Cyps), FK506-binding proteins (FKBPs), and parvulins resulting in a total of 35 human members all together. The proteome-wide PPIase-scan peptide microarray contains the complete primary structure of all human PPIases in the form of overlapping peptide scans. The peptide probes have been synthesized by SPOT technology (13, 14) as amino-oxy-acetyl derivatives enabling chemoselective and covalent immobilization onto appropriately modified glass slides. These include the following FKBPs, cyclophilins, and parvulins resulting in 3,320 peptides: (listed in brackets are the corresponding SwissProt accession numbers followed by the length of the PPIase-derived peptides and the number of overlapping amino acid residues). As we were especially interested in the posttranslational modification of the low molecular weight PPIase, we decided to synthesize peptide with more overlap for these PPIases. FKBP12 (P62942, 15/9), FKBP12.6 (P68106, 15/9), FKBP13 (P26885, 15/9), FKBP19 (Q9NYL4, 15/9), FKBP22 (Q9NWM8, 15/9), FKBP23 (Q9Y680, 15/9), FKBP25 (Q00688, 15/9), FKBP36

(O75344, 15/8), FKBP37 (O00170, 15/7), FKBP38 (Q14318, 15/7), FKBP44 (Q9NZN9, 15/7), FKBP51 (Q13451, 15/7), FKBP52 (Q02790, 15/7), FKBP63 (O95302, 15/7), FKBP65 (Q96AY3, 15/7); FKBP135 (Q9Y4DO, 15/3), Cyp18 (P62937, 13/7), Cyp18.1 (Q9H2H8, 13/9), Cyp18.2 (Q9Y536, 13/7), Cyp18.2a (Q9Y3C6, 15/9), Cyp19.2 (O43447, 13/7), Cyp22 (P30405, 13/7), Cyp23 (P23284, 13/7), Cyp23a (P45877, 15/9), Cyp33 (Q9UNP9, 15/9), Cyp35 (Q81XY8, 15/9), Cyp40 (Q08752, 15/9), Cyp57 (Q8WUA2, 15/9), Cyp59 (Q13356, 15/9), Cyp73 (Q96BP3, 15/9), Cyp89 (Q13427, 15/5), Cyp165 (P30414, 15/1), Pin1 (Q13526, 13/7), Par14 (Q9Y237, 13/7), Par17 (Q52M21, 15/1).

All peptides were synthesized with amino-oxy-acetylated *N*-(3-{2-[2-(3-Amino-propoxy)-ethoxy]-ethoxy}-propyl)-succinyl moiety at the N-terminus allowing both optimal presentation of the peptide to the used enzymes and purification of the immobilized peptides by the removal of truncated acetylated by-products (generated by capping with acetic anhydride) during microarray printing (15). All PPIase-derived peptides were printed threefold to one standard industry glass slide resulting in $3 \times 3{,}320 = 9{,}960$ spots on the so-called PPIase-Chip. Additionally, 2,634 control peptides were printed resulting in 12,594 peptides immobilized on the microarray. Peptide microarrays were designed and produced by JPT Peptide Technologies GmbH (Berlin, Germany).

Subsequent to the incubation of these PPIase-Chips with different cell lysates generated phosphopeptides that were detected by fluorescence scanning subsequent to the treatment with phosphate-specific stain Pro-Q Diamond (Invitrogen) which was claimed to bind specifically to peptides containing phosphorylated amino acids residues such as phosphotyrosine, phosphothreonine, and phosphoserine (16, 17). We validated this stain extensively using phosphopeptide microarrays displaying more than 15,000 phosphopeptides derived from human phosphorylation sites (18). The signals found in the fluorescence images can be correlated to appropriate peptide sequences from the corresponding PPIases using spot-recognition software packages like Genepix.

As an example Fig. 2 shows a fluorescence image of a PPIase microarray incubated with HEK lysate followed by the treatment with phosphate-specific stain Pro-Q Diamond.

2. Materials

1. Micropipettes adjustable from 0.5 to 10 µL and from 100 to 1,000 µL (Eppendorf or Gilson) with corresponding plastic tips.
2. Metal trays with cover.

3. Cell culture 25 cm^2 flasks.
4. 1 mL Eppendorf tubes for the preparation of the phosphorylation assay solution.
5. A plastic sheet cut in 1 mm x 3 cm pieces. We used sheets with a thickness of 0.8 mm resulting in a final assay volume of 400 µL.
6. A centrifuge for standard industry glass slides.
7. Spot-recognition software like GenePix Pro 6.0.
8. Blocking buffer: Tris-buffered saline 50 mM Tris–HCl, 150 mM NaCl, pH 8.0, 3% bovine serum albumin, w/vol.
9. Lysis buffer: 20 mM HEPES–NaOH pH 7.5, 350 mM NaCl, 25% Glycerol; 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.6 µM ammonium molybdate, protease inhibitor cocktail.
10. Phosphorylation assay solution:
    - Complete cell lysate supernatant.
    - 5 mM $MnCl_2$.
    - 10 µL 1 mM ATP, 1 mM $MgCl_2$.
    - Adjust to 400 µL total volume with double distilled water.
11. Phosphate buffered saline: 50 mM phosphate, pH 7.5, 150 mM NaCl.
12. Washing buffer 1: 6 M urea, 20 mM Tris–HCl, pH 7.5.
13. Washing buffer 2: 3 M urea, 20 mM Tris–HCl, pH 7.5.
14. Confluent 25 cm^2 cell culture flask with cell line of choice.
15. Fluorescence scanner with a pixel size smaller than 20 µm (see Note 1).
16. ProQ Diamond stain (Invitrogen, Karlsruhe, Germany).
17. ProQ Diamond destain solution or 20% acetonitrile, 50 mM sodium acetate pH 4.0.
18. Double distilled water.

3. Methods

1. Prepare 25 cm^2 confluent cell culture flask with the cell line of choice.
2. For the drug stimulation of the cells, add the stimulating compound in an appropriate concentration for several hours.

3. Block the microarrays with blocking buffer for 60 min at room temperature. Wash with double distilled water (see Notes 2–6).
4. Before you prepare the cell lysate, wash the cells with phosphate buffered saline (at 37°C) and harvest 5×10^6 in a 15 mL falcon tube.
5. For the preparation of the cell lysate, resuspend the cells in 100 µL lysis buffer and incubate for 30 min on ice. Centrifuge the lysate for 15 min at 16,000 × *g* at 4°C.
6. Prepare the phosphorylation assay solution containing:
   - Complete cell lysate supernatant.
   - 5 mM $MnCl_2$.
   - 10 µL 1 mM ATP, 1 mM $MgCl_2$.
   - Adjust to 400 µL total volume with double distilled water.
7. For phosphorylation reaction two slides, one displaying the peptides and another slide without any peptides but coated with blocking buffer for 1 h at room temperature have to be assembled according to Fig. 1 in a sandwich like format (see Notes 7–13). If two peptide microarrays should be screened, the top slide could be another peptide microarray. The two slides are separated by two spacers generated from a plastic sheet (see Fig. 2). The final assay volume will depend on the thickness of these plastic spacers (0.2 mm thickness will result in 100 µL assay volume; we recommend at least 0.8 mm thickness resulting in 400 µL). The sample has to be applied in between the two slides. Therefore, the top slide is shifted about 1 mm to one side (see Fig. 2). If the pipette tip is adjusted on the position directly over the uncovered bottom slide, the capillary forces allow proper distribution of the sample solution without the formation of air bubbles.
8. The phosphorylation reaction should be performed at 37°C for 3–4 h.
9. Remove the plastic spacers and rinse the peptide microarray with double distilled water, followed by washing with washing buffer 1 and washing buffer 2, Tris-buffered saline, and double distilled water, 20 min each.
10. Incubate microarray with ProQ Diamond stain solution for 30 min (see Notes 14–16).
11. Wash microarray with ProQ Diamond destain solution (three times, 30 min each time).
12. Wash two times 5 min with double distilled water.

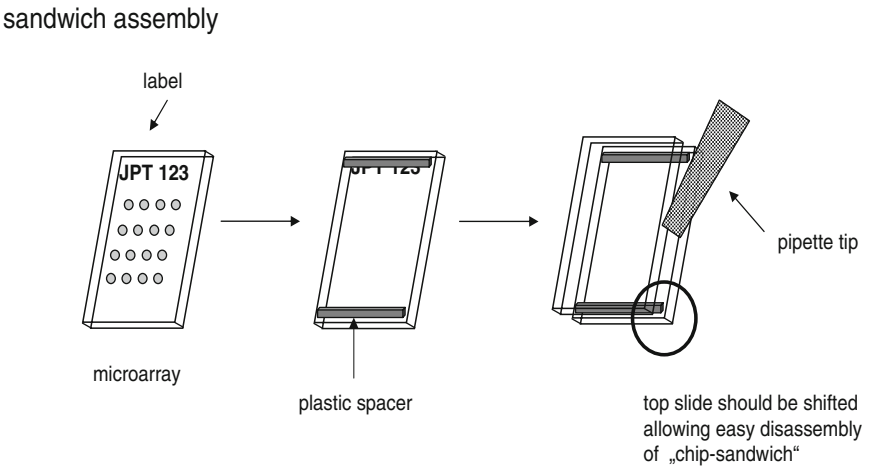


Fig. 1. Assembly of “chip sandwich” is shown. Two plastic spacers are placed between the peptide displaying microarray (*bottom microarray*) and the dummy slide or second peptide displaying microarray (*top microarray*) resulting in a defined reaction chamber. Assay solution is applied via pipette tip into the reaction chamber formed by the two slides. Capillary forces will soak-in the solution without the formation of air bubbles. *Top microarray* is displaced slightly resulting in overlaying ends of the glass slides. This arrangement enables convenient disassembly after the incubation step.

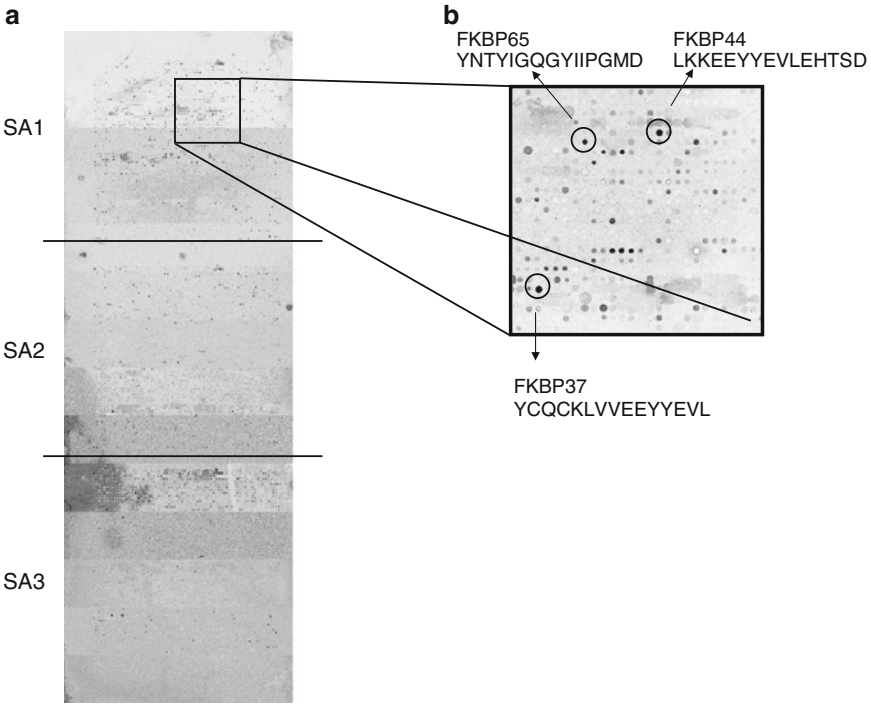


Fig. 2. (**a**) Fluorescence of PPIase-Chip subsequent to incubation with HEK lysate. The phosphorylated peptides were visualized using ProQ Diamond stain. *Black spots* represent peptide spots which are fluorescently labeled by ProQ Diamond stain. The *vertical, solid line* separates the two identical subarrays. (**b**) Enlarged region within one subarray which was printed by one needle. Three phosphorylated peptides are marked by a *circle*. The corresponding peptides, together with the PPIase the sequence was derived from, have been indicated.

13. Dry microarray using a microarray centrifuge. Alternatively, drying using a stream of oil-free nitrogen or argon can be applied.
14. Scan peptide microarray at appropriate laser/filter settings (excitation wavelength: 532 nm, emission wavelength: 590 nm) using microarray fluorescence scanner (see Notes 17 and 18).
15. Use spot-recognition software to get mean of fluorescence intensities of all pixels for each peptide spot.
16. Calculate the mean of background corrected signal intensities for identical peptides (replicates on the microarray).

4. Notes

1. Pixel sizes smaller than 20 μm will result in more accurate data points. We used an Axon4000 scanner at 10 μm resolution.
2. Always handle peptide microarray slides with care. They are made of glass and have sharp edges.
3. Never touch the peptide microarray surface.
4. Always wear powder-free laboratory gloves when handling peptide microarray slides.
5. Hold peptide microarray slides at the edge, which carries the engraved data label. This label provides unique identification of the array.
6. Please take care when dispensing solutions onto the slide surface. Make sure not to contact the surface with pipette-tips or dispensers.
7. Apply the sample to the peptide displaying the side of the glass slide. For JPT peptide microarrays, this is the side with the engraved label.
8. We strongly recommend the arrangement of the two slides during incubation as shown in Fig. 2 (circled region bottom). The slight shift of the top slide compared to the bottom slide allows easy disassembly of the two glass slides.
9. Never whisk the surface of the peptide microarray slide with a cloth.
10. Never use other chemicals than those described. Inappropriate chemicals may destroy the silanol bond formed between the peptides and the modified glass surface.
11. Filter all solutions for the washing steps through 2 μm, preferably 0.45 μm particle filters before use.

12. All plastic equipment which will contact your kinase-activity-containing solution (pipette tips, plastic tubes), and so forth should be coated for at least 1 h with bovine serum albumin solution (blocking buffer) at room temperature before use. It is important to coat for at least 1 h because that process is time dependent. If coating is not made or if coating time is too short, kinases from your assay solution will stick on uncoated parts of the hydrophobic plastic surfaces and are therefore not available for phosphorylation of microarray-bound peptides. This could result in the complete loss of kinase activity in the final assay, especially if highly diluted kinase activity-containing solutions are used.
13. Avoid dust or other particles during each step of the experiment. Dust particles and resulting scratches will cause artifacts during the final signal readout.
14. For incubation with the fluorescently labeled phosphate-specific stain, it is important to use metal trays with a cover or plastic trays completely covered with aluminum foil as these reagents are sensitive toward light.
15. Control incubations using fluorescently labeled phosphate-specific stain alone should be performed in parallel to the kinase fingerprinting experiment to ensure that found signals are not caused by unspecific binding of the stain to immobilized peptides.
16. In principle, phosphorylated proteins from the lysate bound to the immobilized peptides could be detected by the phosphate-specific stain, too. To remove such bound proteins, washings with washing buffer 1 and 2 are sufficient. For JPT peptide microarrays, the amine bond between the immobilized peptide derivatives and the reactive function on the modified glass surface will be stable against these harsh washing conditions.
17. Make sure to scan the side of the microarray displaying the peptides.
18. Fluorescence scanning could be very sensitive depending on the used scanner. Avoid any fluorescent impurities/contaminations inside your assay solution or washing solutions. You can easily check for such impurities by incubating and washing a dummy slide with the same solutions followed by fluorescence imaging at the same laser settings.

References

1. Geiss-Friedlander, R., and Melchior, F. (2007) Concepts in sumoylation: a decade on, *Nat Rev Mol Cell Biol 8*, 947–956.
2. Hunter, T. (2007) The age of crosstalk: phosphorylation, ubiquitination, and beyond, *Mol Cell 28*, 730–738.
3. Shenolikar, S. (2007) Analysis of protein phosphatases: toolbox for unraveling cell signaling networks, *Method Mol Biol 365*, 1–8.
4. Zhang, H., Zha, X., Tan, Y., Hornbeck, P. V., Mastrangelo, A. J., Alessi, D. R., Polakiewicz, R. D., and Comb, M. J. (2002) Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs, *J Biol Chem 277*, 39379–39387.
5. Kloda, A., Martinac, B., and Adams, D. J. (2007) Polymodal regulation of NMDA receptor channels, *Channels (Austin) 1*, 334–343.
6. Yu, R. K., and Bieberich, E. (2001) Regulation of glycosyltransferases in ganglioside biosynthesis by phosphorylation and dephosphorylation, *Mol Cell Endocrinol 177*, 19–24.
7. Surmacz, T. A., Bayer, E., Rahfeld, J. U., Fischer, G., and Bayer, P. (2002) The N-terminal basic domain of human parvulin hPar14 is responsible for the entry to the nucleus and high-affinity DNA-binding, *J Mol Biol 321*, 235–247.
8. Lu, P. J., Zhou, X. Z., Liou, Y. C., Noel, J. P., and Lu, K. P. (2002) Critical role of WW domain phosphorylation in regulating phosphoserine binding activity and Pin1 function, *J Biol Chem 277*, 2381–2384.
9. Wiegand, S., Dakic, B., Rath, A. F., Makarova, G., Sterz, C., Meissner, W., Bette, M., Adamkiewicz, J., Muller-Brusselbach, S., Muller, R., Werner, J. A., and Mandic, R. (2009) The rotamase Pin1 is up-regulated, hypophosphorylated and required for cell cycle progression in head and neck squamous cell carcinomas, *Oral Oncol 45(10)*, e140–e149.
10. Reimer, T., Weiwad, M., Schierhorn, A., Ruecknagel, P. K., Rahfeld, J. U., Bayer, P., and Fischer, G. (2003) Phosphorylation of the N-terminal domain regulates subcellular localization and DNA binding properties of the peptidyl-prolyl cis/trans isomerase hPar14, *J Mol Biol 330*, 955–966.
11. Kay, J. E. (1996) Structure-function relationships in the FK506-binding protein (FKBP) family of peptidylprolyl cis–trans isomerases, *Biochem J 314(Pt 2)*, 361–385.
12. Fanghanel, J., and Fischer, G. (2004) Insights into the catalytic mechanism of peptidyl prolyl cis/trans isomerases, *Front Biosci 9*, 3453–3478.
13. Frank, R. (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports – principles and applications, *J Immunol Methods 267*, 13–26.
14. Reineke, U., Volkmer-Engert, R., and Schneider-Mergener, J. (2001) Applications of peptide arrays prepared by the SPOT-technology, *Curr Opin Biotechnol 12*, 59–64.
15. Reineke, U. and Schutkowski, M. (2006) Peptide arrays in proteomics and drug discovery, in BioMEMS and Biomedical Nanotechnology, Volume II: Micro and Nano-Technologies for Genomics and Proteomics, Springer Science+Business Media, LLC, New York, NY, pp. 161–282.
16. Martin, K., Steinberg, T. H., Cooley, L. A., Gee, K. R., Beechem, J. M., and Patton, W. F. (2003) Quantitative analysis of protein phosphorylation status and protein kinase activity on microarrays using a novel fluorescent phosphorylation sensor dye, *Proteomics 3*, 1244–1255.
17. Steinberg, T. H., Agnew, B. J., Gee, K. R., Leung, W. Y., Goodman, T., Schulenberg, B., Hendrickson, J., Beechem, J. M., Haugland, R. P., and Patton, W. F. (2003) Global quantitative phosphoprotein analysis using Multiplexed Proteomics technology, *Proteomics 3*, 1128–1144.
18. Zerweck, J., Masch, A., and Schutkowski, M. (2009) Peptide microarrays for profiling of modification state-specific antibodies, *Methods Mol Biol 524*, 169–180.

Chapter 15

A Peptide Microarray for Detecting Protein Kinase Activity in Cell Lysates

Xiaoming Han and Yoshiki Katayama

Abstract

Protein kinases (PKs) are widely recognized as valuable targets for disease diagnosis and drug discovery. For this reason, we have developed a sensitive peptide microarray for detecting intracellular PK activity. Peptides are immobilized on a glutaraldehyde-premodified high-amino terminal glass slide, by spotting 2 nL volumes of substrate peptide solutions with an automated microarray spotter. After the peptides are phosphorylated by cell lysates, phosphorylation is specifically recognized by a fluorescence-labeled antiphosphotyrosine antibody for tyrosine kinases, or Phos-tag biotin (a biotinylated phosphate-specific ligand based on Zn^{2+} complex), which is subsequently bound with fluorescence-labeled streptavidin, for serine/threonine kinases. The fluorescence signal is then detected by an automatic microarray scanner. The peptide microarray system involves simple peptide immobilization, requires low sample volumes and provides a high density array. Importantly, it provides high sensitivity for detecting PK activities in cell lysates. Thus, the peptide microarray system is expected to be useful for a high-throughput kinase assay to investigate intracellular kinase activity and has potential applications in disease diagnosis and drug discovery.

Key words: Protein kinase, Peptide, Microarray, High-throughput screening, Cell lysate, Drug discovery

1. Introduction

Protein kinases (PKs) catalyze protein phosphorylation by transferring a phosphate group from adenosine-5′-triphosphate (ATP) to tyrosine, serine, or threonine residues of substrate proteins. Phosphorylation events regulate many important intracellular processes, including cell differentiation, adhesion, proliferation, and apoptosis (1). Dysfunction in PK activity is proven to be a key factor in causing many diseases, such as cancer, inflammation, cardiovascular diseases, and diabetes. Therefore, PKs are widely

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_15, © Springer Science+Business Media, LLC 2010

recognized as valuable targets for disease diagnosis and drug discovery (2, 3). However, traditional methods for detecting PK activity, such as filter-binding assays, gel-based assays, and enzyme-linked immunosorbent assays (ELISAs) are restricted by their low throughputs and complex procedures. Recently, high-throughput microarrays have been extensively developed to detect PK activity (4). Peptides, as the probes used in the microarrays, will likely be more reliable than proteins because they are more stable and are easily prepared and handled. However, the study of intracellular PK activity on peptide microarrays requires improved workflows that confer good sensitivities and reduce nonspecific protein adsorption (5, 6).

We have developed a sensitive peptide microarray system for detecting intracellular PK activity by a comprehensive optimization of every step for construction and detection (7–9). Our experimental protocol is illustrated in Fig. 1. In this protocol, a high-density amino terminal glass is pretreated with glutaraldehyde to obtain an aldehyde surface. One aldehyde group of glutaraldehyde reacts with one amine groups on the glass to form a Schiff base, leaving the other aldehyde group unreacted. Subsequently, cysteine-terminal peptides are automatically delivered

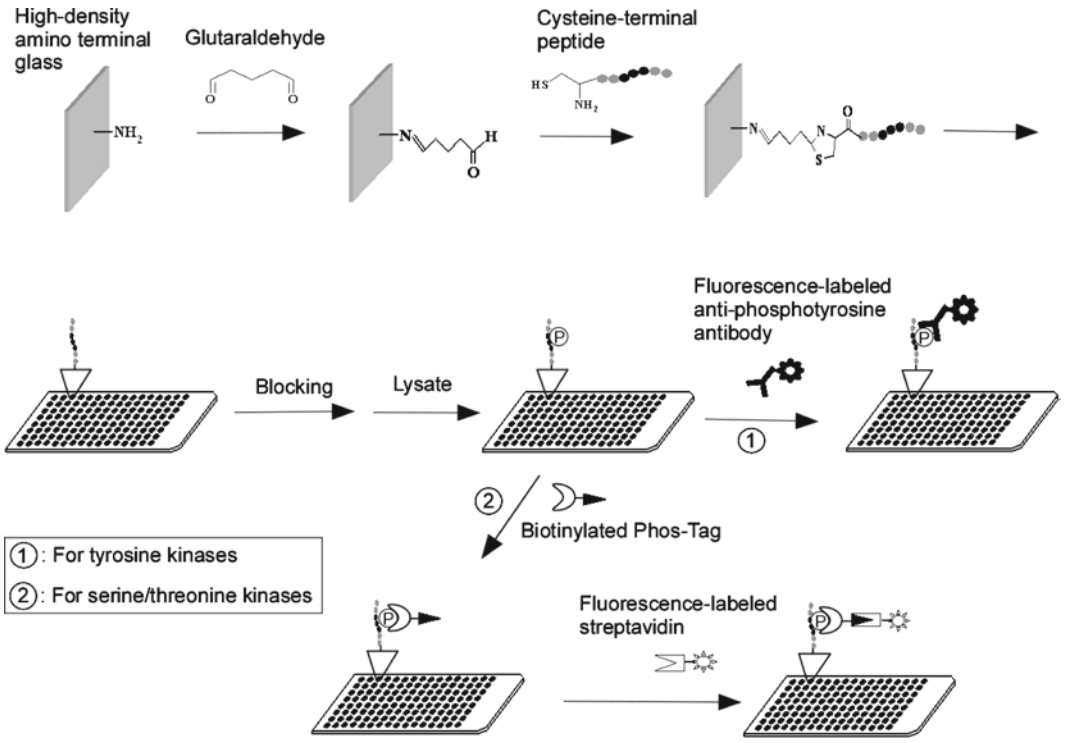


Fig. 1. Schematic experimental protocol to detect PK activity in cell lysates using a peptide microarray.

onto the glass using a microarray spotter. The peptides are then covalently immobilized onto the glass via formation of five-membered thiazolidine rings between the cysteine at the amino terminus of the peptides and the free aldehyde group of the immobilized glutaraldehyde. The peptide-tethered glass is then blocked by Blocking One P (a commercial blocking agent) to avoid nonspecific adsorption in the following phosphorylation reaction. Cell lysates are prepared and used to phosphorylate the on-chip peptides. The phosphorylation is then recognized by a fluorescence-labeled antiphosphotyrosine antibody for tyrosine kinases or a Phos-tag biotin (a biotinylated phosphate-specific ligand), which is subsequently bound with fluorescence-labeled streptavidin for serine/threonine kinases. The fluorescence signal is detected by an automatic microarray scanner. The peptide microarray system involves simple peptide immobilization, requires low sample volumes and presents a high density array. Importantly, it provides high sensitivity to detect PK activity in cell lysates. Thus, the peptide microarray system is expected to be useful for a high-throughput kinase assay to monitor intracellular kinase activity and has potential applications in disease diagnosis and drug discovery.

2. Materials

2.1. Peptide Preparation

1. Reagents for peptide synthesis: Rink amide-AM resin (0.62 mmol/g) and Fmoc amino acids (Novabiochem, Darmstadt, Germany); 2-(1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethyl-uronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), *N*-ethyldiisopropylamine (DIEA), Piperidine (*Toxic and corrosive*), and *N*-methylpyrrolidone (NMP) (Watanabe, Hiroshima, Japan); dimethylformamide (DMF) (Kanto Chemical, Tokyo, Japan); methanol (MeOH).
2. APEX 396 Multiple Peptide Synthesizer (Advanced ChemTech, KY, USA).
3. Reagents for peptide purification: trifluoroacetic acid (TFA) (*Toxic and corrosive*) and 1, 2-ethanedithiol (EDT) (Wako Pure Chemical, Osaka, Japan); triisopropylsilane (TIS) (Tokyo Kasei Kogyo, Tokyo, Japan); acetonitrile and diethyl ether (Kanto Chemical).
4. Reverse-phase liquid chromatography equipped with an electrospray mass spectrometer detector (Micromass Platform II connected to Waters Alliance HPLC system using a Phenomenex LUNA C18 column (2.1 × 50 mm)) (Waters, Milford, USA).

2.2. Cell Culture and Lysate Preparation

1. Cell culture medium for A431 cells: Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, New York, USA) and 1% antibiotic–antimycotic (Gibco, CA, USA).
2. Cell stimulation: SU6656 (Amersham Biosciences, Bucks, UK) is dissolved to 5 mM in dimethyl sulfoxide (DMSO) (Wako) and stored at –80°C. 5 mM SU6656 is diluted to 2 μM with FBS-free DMEM containing 1% antibiotic–antimycotic. Dulbecco's phosphate-buffered saline (D-PBS) (Gibco).
3. Cell lysis buffer A (*Prepared fresh as required*): 10 mM potassium phosphate (pH 7.25), 1 mM ethylene diamine tetraacetic acid (EDTA), 5 mM ethylene glycol tetraacetic acid (EGTA), 10 mM magnesium chloride ($MgCl_2$), 2 mM Dithiothreitol (DTT), 1 mM sodium orthovanadate, 80 mM β-glycerophosphate (Fluka, Steinheim, Germany), 3 μg/mL pepstatin A (Calbiochem, Darmstadt, Germany), 5 μg/mL aprotinin (Sigma-Aldrich, St. Louis, USA), 1 mM phenylmethylsulfonyl fluoride (Nacalai Tesque). Stored at 4°C.
4. Cell lysis buffer B (*Prepared fresh as required*): Cell lysis buffer A +0.2% triton X-100. Stored at 4°C.
5. Ultrasonic homogenizer Model 450 (Branson, Danbury, US).
6. BCA™ Protein Assay Kit (Pierce, Rockford, USA).
7. Cell culture medium for HepG2 cells: Minimum essential medium (MEM) (Wako) supplemented with 10% FBS and 1% antibiotic–antimycotic.

2.3. Mouse Tumor Inoculation and Tissue Lysate Preparation

1. Male 5-week-old BALB/c nu/nu mouse (Kyudo, Saga, Japan); Hanks' balanced salt solution (Gibco).
2. Digital homogenizer (Iuchi, Osaka, Japan).

2.4. Fabrication of a Peptide Microarray

1. High-density amino terminal glass slides (Matsunami Glass, Osaka, Japan) (see Note 1). Stored at room temperature (RT).
2. 50 mM $NaHCO_3$ (pH 9.5). Stored at RT.
3. Glutaraldehyde (Wako). Stored at 4°C.
4. Tris-(2-carboxyethyl)phosphine hydrochloride (Promega, Wisconsin, USA) (*Corrosive; Protect from light and moisture*). Stored at 4°C.
5. Microarray spotter (Kaken Genetics, Chiba, Japan).
6. TBS-T: 25 mM Tris-buffered saline (TBS) containing 0.05% Tween 20 (pH 7.4). Stored at RT.

2.5. On-Chip Phosphorylation

1. Blocking One-P (5X) (Nacalai Tesque). Stored at 4°C.
2. Super PAP pen (DAIDOSANGYO, Tokyo, Japan) (see Note 2).
3. Phosphorylation solution (*Prepared fresh as required*): 15 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.01% Tween 20, 5 mM DTT, 0.1 mM Adenosine-5′-triphosphate disodium salt hydrate (ATP) (*Prepared fresh as required*) (Sigma-Aldrich), 100 μg/mL cell lysate (as a starter regeant).
4. TBS-S: 25 mM TBS containing 1% sodium dodecyl sulfate (SDS), pH 7.4. Stored at RT.

2.6. Phosphorylation Recognition

1. Probe for phosphotyrosine: Cy5-conjugated antiphosphotyrosine antibody (*Protect from light*) (Amersham Biosciences). Stored at 4°C.
2. Microarray scanner (Packard Bioscience, Billerica, USA).
3. Probe for phosphoserine/threonine: Phos-tag biotin (Toyobo, Osaka, Japan); Dylight™ 649-conjugated streptavidin (*Protect from light*) (Pierce Biotechnology, Rockford, USA). Stored at 4°C.
4. Phos-tag buffer: 10 mM Hepes-NaOH (HEPES) (pH 7.3), 1 mM zinc nitrate ($Zn(NO_3)_2$), 0.005% Tween 20, 10% ethanol, 0.2 M sodium nitrate ($NaNO_3$). Stored at 4°C.

3. Methods

3.1. Peptide Preparation

1. Synthesize peptides using the APEX 396 Multiple Peptide Synthesizer with Fmoc chemistry. Use Rink Amide AM resins as the solid supports and a standard HOBT/HBTU/DIEA coupling method throughout the process. Load each microreactor with 16.2 mg of resins (0.01 mmol). Swell the resins thoroughly in 1.3 mL of DMF for 1 h with mixing at 600 rpm. Drain DMF out of the microreactor. Transfer 0.5 mL of 20% piperidine in DMF to the microreactor and mix the solution for 10 min at 600 rpm for Fmoc deprotection. Drain the solution out of the microreactor and then wash the resins three times for 1 min with 1.3 mL of DMF at 600 rpm. Transfer 0.8 mL of 0.04 mmol Fmoc amino acid (4.0 eq.) in NMP containing 0.5 M HOBT, 0.25 mL of 0.5 M HBTU in DMF, and 0.25 mL of 1 M DIEA in DMF to the microreactor for coupling. Mix the solution for 20 min at 600 rpm. Drain the solution out of the microreactor. Repeat the coupling process twice to increase the coupling efficiency. Wash the resins for 1 min three times

with 1.3 mL of DMF at 600 rpm. Repeat the same steps for each cycle. Finally, wash the resins three times for 1 min with 1.3 mL of MeOH at 600 rpm. Drain the MeOH solution.

2. Dry the resins overnight in air. Transfer 1 mL of a cleavage cocktail containing 94% TFA, 2.5% water, 2.5% 1,2-ethanditiol and 1% triisopropylsilane to the microreactor. Mix the solution with the resins for 1 h at 600 rpm. Collect the solution in a 15 mL centrifuge tube. Add 9 mL of cold diethylether to the centrifuge tube for the precipitation of the cleaved peptides. Separate the peptides by centrifugation at 3,000 *g* for 5 min at 4°C and remove the supernatant. Wash the peptides with 5 mL of cold diethylether two more times. Dry the peptides completely under a flow of N_2. Dissolve the dried peptides in 1 mL of water. Purify the obtained crude peptides by the reverse-phase liquid chromatography using a linear gradient of 10–90% acetonitrile containing 0.1% TFA at a flow rate of 3.0 mL/min. Lyophilize the purified peptides to a white powder.

3.2. Cell Culture and Lysate Preparation

1. Culture A431 cells (10^7 cells) in a confluent layer in 10 cm dishes in a humidified atmosphere containing 5% CO_2 at 37°C.
2. Replace the cell culture medium with 10 mL of serum-free DMEM containing 1% antibiotic–antimycotic and incubate the cells for 18 h before drug stimulation. Stimulate the cells by 2 μM SU6656, a Src-specific inhibitor (10), for 2 h (see Note 3). Wash the cells twice with 5 mL of cold D-PBS. Add 1 mL of D-PBS to the cells and collect them by scraping. Transfer the cell containing solution into a 1.5 mL tube. Centrifuge the solution at 1,500 rpm for 5 min at 4°C. Remove the supernatant.
3. Add 200 μL of the cell lysis buffer A to the tube to resuspend the cells. Sonicate the cells for 15 s three times in an ice bath (see Note 4) using the ultrasonic homogenizer. Centrifuge the homogenate at 100,000 *g* for 60 min. Collect the supernatant as "cytosol fractions." Resuspend the remaining pellet in 200 μL of cell lysis buffer B. Sonicate the pellet for 15 s three times in an ice bath. Centrifuge the solution at 100,000 *g* for 40 min. Finally, collect the supernatant as "membrane fractions." Use the membrane fractions for Src experiments because Src is mainly located adjacent to the membrane.
4. Determine the protein concentrations in the cell lysates using the BCA™ protein assay kit. The protein concentrations of cytosol and membrane fractions are about 1 mg/mL.

5. Culture HepG2 cells (10^7 cells) in a confluent layer in 10 cm dishes in a humidified atmosphere containing 5% CO_2 at 37°C. Prepare the HepG2 cell lysate using the same method as preparation of A431 cell lysate. Use the cytosol fractions to detect serine/threonine kinase activities.

3.3. Mouse Tumor Inoculation and Tissue Lysate Preparation

1. Perform all animal studies in accordance with the Guidelines for Animal Experiments of Kyushu University. Inoculate 10^7 A431 cells subcutaneously in 100 μL of Hanks' balanced salt solution to male 5-week-old BALB/c nu/nu mice. Allow the tumor to grow to about 8 mm in diameter. Sacrifice the mice by decapitation. Excise the tumor and subcutaneous skin tissue.
2. Homogenize the tissues gently in 1 mL of cell lysis buffer A by the digital homogenizer. Centrifuge the homogenate at 1,000 *g* at 4°C for 5 min and remove the supernatant. Wash the precipitate with 1 mL of cell lysis buffer A by pipetting. Centrifuge the suspended solution at 1,000 *g* at 4°C for 5 min and remove the supernatant. Resuspend the precipitate in 0.5 mL of cell lysis buffer A. Follow the steps for preparation of A431 cell lysates as described in Subheading 3.2 in step 3.

3.4. Fabrication of a Peptide Microarray

1. Wash a high amino-modified glass slide in a glass container of pure water for 5 min with sonication. Dry the slide under the N_2 flow.
2. Prepare 1% glutaraldehyde as follows: add 9.5 mL of 50 mM $NaHCO_3$ (pH 9.5) into a glass dish and subsequently, add 0.5 mL of 20% glutaraldehyde. Mix the solution gently. Place the glass slide into the solution, immerse it (see Note 5) and incubate the slide at 37°C for 2 h. Rinse the glass slide twice with pure water for 8 min with sonication. Dry the slide under the N_2 flow (see Note 6).
3. Prepare 20 μL of peptide solutions containing 50 μM peptide and 50 μM TCEP in 50 mM $NaHCO_3$ (pH 9.5) in a 384 well plate. Leave the peptide solution at 4°C for 20 min for reduction of the disulfide bond.
4. Spot the peptide solutions onto the slide with φ150 μm pins using the microarray spotter. Prepare two peptide grids containing the same peptides (one domain for control). Incubate the slide in a humid glass dish at RT overnight. Rinse the slide twice with TBS-T for 4 min with sonication. Dry the slide w under the N_2 flow.

3.5. On-Chip Phosphorylation

1. Prepare 10 mL of blocking solution as follows: add 8 mL of TBS-T into a glass dish, followed by adding 2 mL of Blocking

One-P. Mix the solution gently. Immerse the slide in the solution (see Note 5) and incubate the slide for 1 h at RT. Rinse the slide twice with TBS-T for 8 min with sonication and once with pure water for 3 min with sonication. Dry the slide under the N_2 flow. Circle the peptide grids on the slide using the Super PAP pen.

2. Drop 150 μL of the phosphorylation solution onto the peptide grids on the slide using a pipette (see Note 7). Incubate the slide in a humid glass dish at 37°C for 2 h. Rinse the slide three times with TBS-S for 8 min with sonication and once with pure water for 3 min with sonication. Dry the slide under the N_2 flow.

3.6. Phosphorylation Recognition

3.6.1. Detection of Phosphotyrosine

1. Prepare 300 μL of 3.33 μg/mL Cy5 antiphosphotyrosine antibody by adding 1 μL of 1 mg/mL antibody to 299 μL of TBS-T. Vortex the solution. Drop 150 μL of the solution separately onto the peptide grids (see Note 7) and incubate the slide in a humid atmosphere at RT for 20 min. Rinse the slide once with TBS-T for 5 min with sonication followed by flushing with pure water. Dry the slide under the N_2 flow. Scan the slide with the microarray scanner. The results using the Src peptides in Table 1 for the detection of the tyrosine kinase, Src, activity in A431 cells (Fig. 2) and mouse tumor (Fig. 3) are shown.

3.6.2. Detection of Phosphoserine/Threonine

1. Recognition of phosphoserine/threonine using Phos-tag biotin: Prepare 300 μL of 10 μg/mL Phos-tag biotin solution by diluting 1 mg/mL Phos-tag biotin with Phos-tag buffer. Drop 150 μL of the 10 μg/mL Phos-tag biotin solution onto the peptide grids (see Note 7) and incubate the slide in a humid atmosphere at RT for 60 min. Flush the slide with pure water and dry it under the N_2 flow.

Table 1
Src peptides. The underlined amino acid indicates the phosphorylation site

Name	Peptide sequence	Comment
2-pY	H–CXXEEI*p*<u>Y</u>GEFD–NH_2	Tyr-phosphorylated (positive control)
2-Y	H–CXXEEI<u>Y</u>GEFD–NH_2	Substrate
2-F	H–CXXEEI*F*GEFD–NH_2	Phe-substituted (negative control)

X = –NH–$(CH_2CH_2O)_2$–CH_2CO–

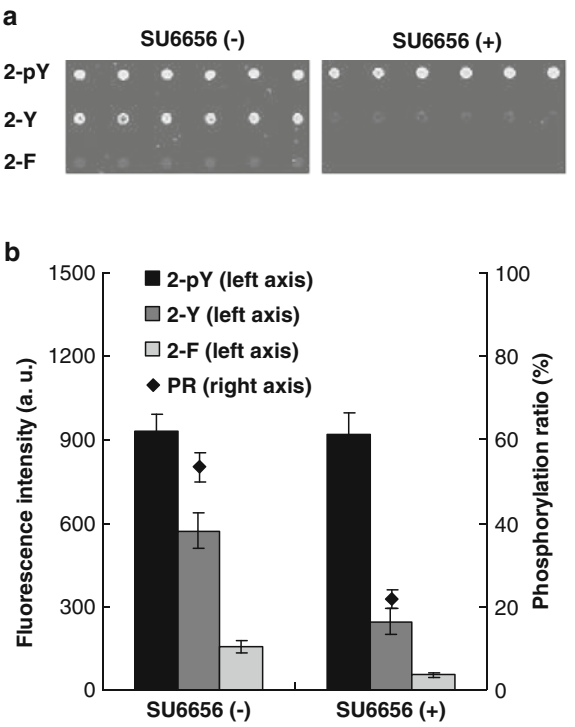


Fig. 2. Detection of Src activity in A431 cells with (SU6656 (+)) or without (SU6656 (–)) SU6656 stimulation using a peptide microarray. (**a**) Fluorescence image of the peptides (positive control 2-pY, substrate 2-Y, and negative control 2-F). The distance between two spots is 1,000 μm. (**b**) Fluorescence intensity of the peptides (*left axis*) and phosphorylation ratio of the substrate (*right axis*). The phosphorylation ratio is calculated from the following equation: Phosphorylation ratio (%) = (FI of substrate – FI of negative control)/(FI of positive control – FI of negative control), where FI represents the fluorescence intensity. Data points are the means of six replicates and error bars represent the standard deviation. With SU6656 stimulation, the fluorescence intensity and phosphorylation ratio of the substrate were both inhibited. This demonstrates that the inhibition of Src activity in A431 cell by SU6656 is successfully detected by the peptide microarray.

2. Recognition of biotin using Dylight™ 649-conjugated streptavidin: Prepare 300 μL of 2 μg/mL Dylight™ 649-conjugated streptavidin by diluting 1 mg/mL Dylight™ 649-conjugated streptavidin with 10 mM HEPES containing 1/20 Blocking One-P. Drop 150 μL of the 2 μg/mL Dylight™ 649-conjugated streptavidin onto the peptide grids (see Note 7) and incubate the slide in a humid atmosphere at RT for 30 min. Rinse the slide once with TBS-T for 2 min with sonication and then flush it with pure water. Dry the slide and scan it with the microarray scanner. A sample fluorescence image of 804 types of peptides used to detect serine/threonine kinase activities in HepG2 cells is shown in Fig. 4.

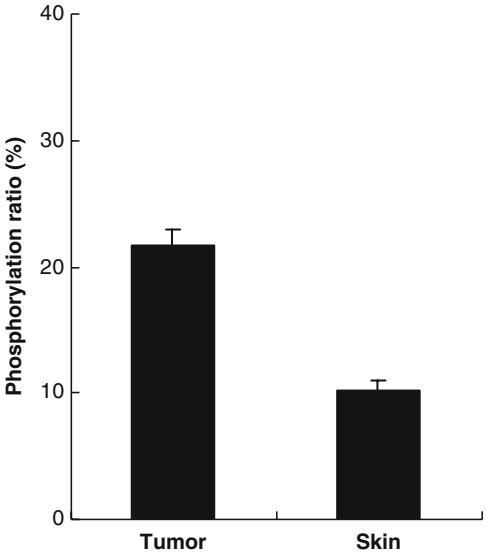


Fig. 3. Detection of Src activity in A431-bearing mouse tumor and normal skin tissue using a peptide microarray. Data points are the means of six replicates and error bars represent the standard deviation. The Src activity in the tumor is about two times higher than that in normal skin tissue. This indicates that Src activity in animal tissues could be detected using the peptide microarray.

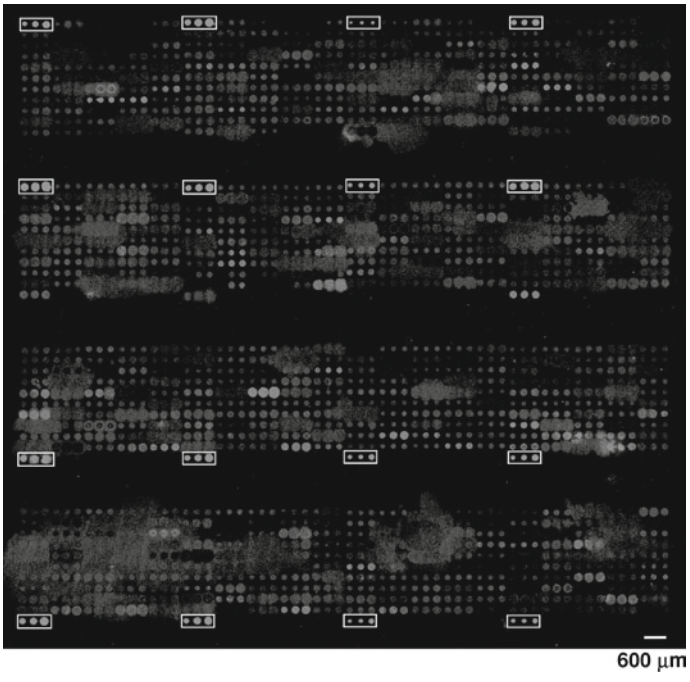


Fig. 4. Fluorescence image of 804 types of peptides used to detect serine/threonine kinase activities in HepG2 cells. The 804 types of peptides were spotted in triplicate on the glass (a total of 2,412 spots). The circled spots are positive controls that have a phosphothreonine residue. The distance between two spots is 300 μm and the scale bar on the bottom represents 600 μm.

4. Notes

1. The high-density amino terminal glass provides more than two times larger amino group density (12 units/nm^2) than that of APTES (3-aminopropyltriethoxysilane)-modified glass (5 units/nm^2) (11), enabling much higher peptide immobilization for sensitive detection.
2. The ink of the pen is water-insoluble and has no effect on reactions. The ink is also resistant to the detergents contained in TBS-T and TBS-S. Reaction solutions that are dropped onto the circled area could be held without any spread.
3. The final concentration of DMSO used to dissolve SU6656 should be less that 0.1%. Thus, the effect of DMSO can be considered negligible.
4. Three 15 s sonications are better than one treatment for 45 s because with a 45-s sonication treatment, the tube would increase in temperature. After every 15 s, the tube should be cooled down with ice to avoid protein denaturization.
5. The slide should be totally immersed.
6. Water drops and moisture should be totally eliminated. Otherwise, the spot will spread when the peptide solution is spotted onto the surface.
7. The peptide-spotted area should be totally covered by the solution. When the solution is dropped by a pipette, the pipette tip should not contact the glass surface.

Acknowledgment

This work was supported by New Energy and Industrial Technology Development Organization (NEDO) and Japan Science and Technology Agency (JST).

References

1. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science* **298**, 1912–34.
2. Olive, D. M. (2004) Quantitative methods for the analysis of protein phosphorylation in drug development. *Expert Rev. Proteomics* **1**, 327–41.
3. Fabbro, D., Ruetz, S., Buchdunger, E., Cowan-Jacob, S. W., Fendrich, G., Liebetanz, J., Mestan, J., O'Reilly, T., Traxler, P., Chaudhuri, B., Fretz, H., Zimmermann, J., and Meyer, T. (2002) Protein kinases as targets for anticancer agents: from inhibitors to useful drugs. *Pharmacol. Ther.* **93**, 79–98.
4. Uttamchandani, M., Wang, J., and Yao, S. Q. (2006) Protein and small molecule microarrays: powerful tools for high-throughput proteomics. *Mol. BioSyst.* **2**, 58–68.

5. Horiuchi, K. Y., Wang Y., Diamond, S. L., and Ma, H. (2006) Microarrays for the functional analysis of the chemical-kinase interactome. *J. Biomol. Screen.* **11**, 48–56.
6. Houseman, B. T., Huh, J. H., Kron, S. J., and Mrksich, M. (2002) Peptide chips for the quantitative evaluation of protein kinase activity. *Nat. Biotechnol.* **20**, 270–74.
7. Shigaki, S., Yamaji, T., Han, X., Yamanouchi, G., Sonoda, T., Okitsu, O., Mori, T., Niidome, T., and Katayama, Y. (2007) A peptide microarray for the detection of protein kinase activity in cell lysate. *Anal. Sci.* **23**, 271–75.
8. Han, X., Shigaki, S., Yamaji, T., Yamanouchi, G., Mori, T., Niidome, T., and Katayama, Y. (2008) A quantitative peptide array for evaluation of protein kinase activity. *Anal. Biochem.* **372**, 106–15.
9. Han, X., Yamanouchi, G., Mori, T., Kang, J-H., Niidome, T., and Katayama, Y. (2009) Monitoring protein kinase activity in cell lysates using a high density peptide microarray. *J. Biomol. Screen.* **14**, 256–62.
10. Blake, R. A., Broome, M. A., Liu, X. D., Wu, J. M., Gishizky, M., Sun, L., Courtneidge, S. A. (2000) SU6656, a selective Src family kinase inhibitor, used to probe growth factor signaling. *Mol. Cell Biol.* **20**, 9018–27.
11. Moon, J. H., Shin, J. W., Kim, S. Y., and Park, J. W. (1996) Formation of uniform aminosilane thin layers: an imine formation to measure relative surface density of the amine group. *Langmuir* **12**, 4621–24.

Chapter 16

Chemical Microarrays Constructed by Selective Attachment of Hydrazide-Conjugated Substances to Epoxide Surfaces and Their Applications

Sungjin Park, Myung-Ryul Lee, and Injae Shin

Abstract

Microarray technology has received considerable attention for rapid analysis of biomolecular interactions and high-throughput screening to identify binding partners. An efficient and selective immobilization technique of substances on the surface is essential for successful construction of microarrays. Although a variety of immobilization methods have been exploited to prepare microarrays over the past decade, a superior technique needs to be developed for diverse applications. Recently, an efficient and simple method that relies on selective reactions between the hydrazide conjugated to substances and the epoxide derivatized on the solid surface was developed to fabricate chemical microarrays. Reactions between hydrazides with epoxides are highly selective in that they take place even in the presence of other potent nucleophiles such as amines and thiols. This technique is utilized to immobilize various substances such as small molecules, carbohydrates, and peptides to glass surfaces. The microarrays constructed by this immobilization method are used to evaluate protein binding to carbohydrates, peptides, and small molecules. In addition, the microarrays are also employed to determine binding affinities between proteins and binding partners as well as profiling of enzyme activities.

Key words: Carbohydrates, Chemoselectivity, Dissociation constants, Enzyme activity, High-throughput screening, Immobilization, Microarrays, Peptides, Protein binding, Small molecules

1. Introduction

In the postgenomic era, functional studies of proteins and glycans have been extensively performed for biological and biomedical applications. As powerful tools for these studies, microarray-based technologies, such as DNA, carbohydrate, peptide, and protein microarrays, have been developed for accelerating the identification of lead compounds and for genomic, transcriptomic, glycomic,

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_16,

and proteomic research (1–12). In addition, small molecule microarrays have also been exploited as high-throughput methods to identify bioactive compounds that selectively bind to proteins (13–15). Small molecules that modulate biological processes serve as useful tools in the studies of the functions of proteins as well in the development of pharmaceutical agents. These microarray-based technologies facilitate fast, quantitative and simultaneous assessment of a large number of biomolecular interactions using limited quantities of samples.

Many biologically relevant compounds possess various functional groups such as alcohols (-OH), amines ($-NH_2$), carboxylic acids (CO_2H), and/or thiols (-SH). The major requirement of techniques used to fabricate chemical microarrays is that immobilization of the diversely functionalized compounds to the modified surfaces must be highly selective. As a result, strategies that employ efficient and selective ligation processes would be generally applicable to the preparation of microarrays that contain covalently linked, biologically interesting molecules with diverse functionalities. Several criteria must be met in designing a general method to prepare chemical microarrays. Firstly, the diverse substances possessing specific functional groups that are used for selective reactions with the modified solid surfaces must be easily synthesized by solid-phase synthesis. Secondly, functional groups that selectively react with substances must be easily incorporated on to the solid surfaces. Lastly, the diversely structured and functionalized substances released from a solid support must undergo site-specific covalent immobilization on the modified surfaces. Strategies, which fit these criteria, employ highly chemoselective ligation reactions. The immobilization technique which relies on the use of reactions between hydrazide-containing substances and epoxide-derivatized surfaces is suitable for the fabrication of chemical microarrays (see Fig. 1). The hydrazide groups incorporated into the substances are used as a handle in their solid-phase synthesis. The epoxide-derivatized glass slide is easily created by immersing amine-coated glass slides into a solution of poly(ethylene glycol) diglycidyl ether. Importantly, the reactions between hydrazides and epoxides are chemoselective even in the presence of other functionalities such as thiol and amine groups. This immobilization method has been applied to the efficient construction of microarrays containing small molecules, peptides, and carbohydrates (16–18).

In this chapter, we provide protocols for the fabrication of chemical microarrays based on the immobilization of hydrazide-containing compounds on epoxide-coated glass slides. We also describe the applications to (1) rapidly analyze protein binding to carbohydrates, peptides, and small molecules, (2) determine binding affinities between proteins and binding partners, and (3) profile enzyme activities.

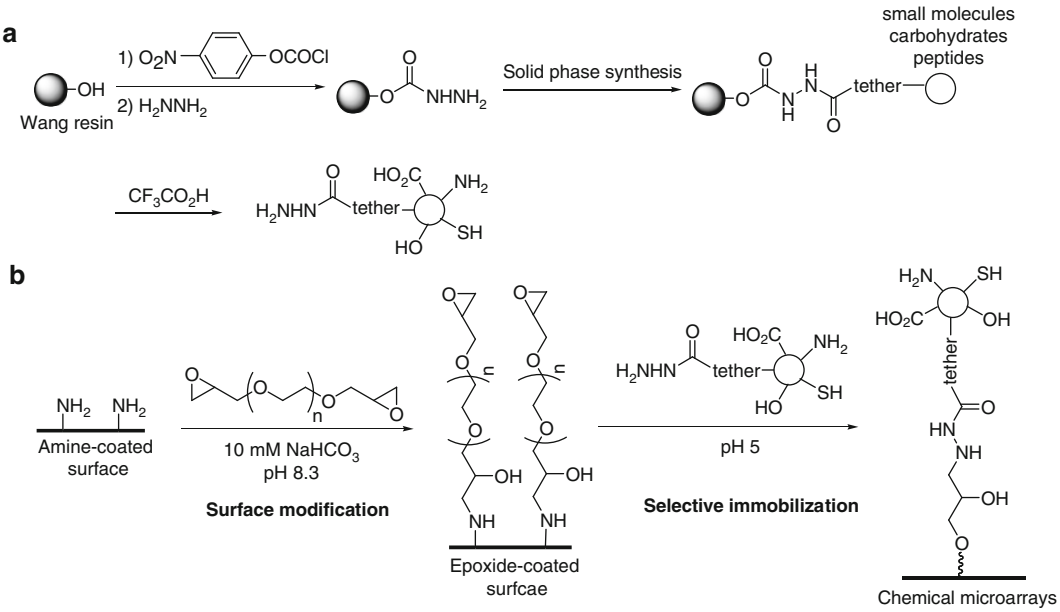


Fig. 1. (**a**) Synthesis of a chemical library on a solid support and (**b**) microarray fabrication based on the immobilization of hydrazide-conjugated substances on epoxide-coated glass slides.

2. Materials

2.1. Fabrication of Chemical Microarrays

1. Hydrazide-conjugated substances are not commercially available and thus should be synthesized according to the known procedures (see Fig. 1) (16–18).
2. Buffers for dissolving compounds: 100 mM sodium phosphate buffer (pH 5.0) containing 40–50% (v/v) glycerol (see Note 1). The hydrazide-conjugated compounds are dissolved at 0.1–5.0 mM in 100 mM sodium phosphate buffer (pH 5.0) containing 40–50% glycerol and then stored at –70°C. If solubility of the probes is poor, dissolve the probes in a minimum amount of DMSO and dilute with buffer containing 40–50% glycerol.
3. Amine glass slides are purchased from commercial suppliers such as TeleChem International, Inc. or Schott Nexterion (see Note 2).
4. 3% Poly(ethylene glycol) diglycidyl ether (Aldrich) in 10 mM sodium bicarbonate (pH 8.3) to prepare epoxide-coated glass slides from amine slides.
5. PBS (pH 7.4) containing 0.1% Tween 20.
6. A plastic film (thickness: 0.1–0.2 mm, B.S. Inc.) that is coated by adhesive at one side.

7. Slide staining tray and slide staining jar (GambH & Co. KG).
8. Adjustable reciprocating orbital shaker (Branstead international).
9. Mini slide centrifuge (TOMY, Japan).
10. MicroSys 5100 microarrayer (Cartisian Technologies) fitted with Stealth Microspotting pins (TeleChem International).

2.2. Labeling of Proteins by Fluorescent Dyes

1. Fluorescein isothiocyanate (FITC) (Aldrich or Molecular Probes) is dissolved at 50 mg/ml in DMSO and stored at –20°C. Since FITC is light-sensitive, keep the solution in the dark.
2. One tube of Cy3- or Cy5-N-hydroxysuccinimide (Cy3- or Cy5-NHS) (Amersham Biosciences) is dissolved in 10 µl DMSO and stored at –20°C. Since Cy3 and Cy5 are light-sensitive, keep the solutions in the dark.
3. Unlabeled proteins are dissolved at 1–10 mg/ml (small proteins: 1–3 mg/ml, large proteins or antibodies: 1–10 mg/ml) in 100 mM sodium bicarbonate (pH 8.0) and stored at 4°C or –20°C (see Note 3). Some fluorescent dye-labeled lectins are commercially available from Vector Laboratories or Sigma.
4. PD-10 desalting column (Amersham Biosciences).

2.3. Applications of Chemical Microarrays

1. PBS (pH 7.4) containing 0.1% Tween 20 and 1% bovine serum albumin (BSA). BSA should be added to the buffer prior to use.
2. Proteins used for analysis of their binding properties.
3. Protein storage buffer: typically PBS buffer (pH 7.4) for lectins.
4. A solution of β-1,4-galactosyltransferase (23 mU) (Calbiochem), 10 mM $MnCl_2$ and 0.1 mM UDP-Gal in 50 mM HEPES buffer, pH 7.5.
5. 35 mM SDS in PBS buffer (pH 7.4).
6. Cartisian AxSys software (Cartisian Technologies).
7. Temperature- and humanity-controlled incubator (Daihan Scientific, Korea).
8. ArrayWorx™ microarray scanner (Applied Precisions, USA).
9. ImaGene 6.1 software and origin Pro7.0 software.

3. Methods

Chemical microarrays are constructed by printing very small quantities (usually 1 nl) of hydrazide-conjugated substances on the epoxide-derivatized glass slides using a robotic printing microarrayer. Substances used for immobilization should be dissolved in the proper buffer containing 40–50% glycerol to suppress undesired

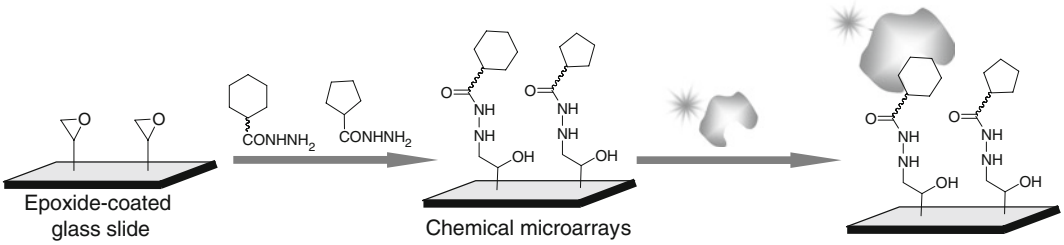


Fig. 2. Fabrication of chemical microarrays and their applications for profiling of proteins.

evaporation of nanodroplets during spotting and immobilization steps. Epoxide-coated glass slides are prepared by immersing amine-coated glass slides into a solution of poly(ethylene glycol) diglycidyl ether (3% solution in 10 mM $NaHCO_3$, pH 8.3). Chemical microarrays fabricated by this method can be used for biological research and biomedical applications. For these studies, the microarrays are incubated with fluorescent dye-labeled proteins, and bound proteins are visualized or quantitated by using a fluorescence scanner (see Fig. 2). A commonly occurring problem in the detection of bound proteins on the microarrays is high background fluorescence due to nonspecific interactions of fluorescent proteins with modified surfaces. This problem can be solved by treating the fabricated carbohydrate microarrays with 1% BSA prior to incubation of the microarrays with labeled proteins. Alternatively, hydrophilic surfaces coated by poly(ethylene glycol) considerably suppress the nonspecific adsorption of proteins on the surfaces.

3.1. Fabrication of Chemical Microarrays

1. Place amine-derivatized glass slides in a slide staining tray and transfer the slides into a staining jar containing 170 ml of a solution of 3% poly(ethylene glycol) diglycidyl ether in 10 mM sodium bicarbonate (pH 8.3).
2. Keep the slides immersed in the staining jar for 0.5–1 h at room temperature with gentle shaking on an orbital shaker (see Note 4).
3. After thorough washing of the slides in the staining jar with 170 ml of deionized water with gentle shaking (10 min × 3), dry epoxide-coated slides by purging with Ar gas or by centrifuging (480 × *g*) for 1.5 min at room temperature using a slide centrifuge (see Note 5). Slides can be stored at room temperature in a desiccator for several weeks.
4. Prepare 100 µl of 0.1–5.0 mM hydrazide-conjugated compound solutions in 100 mM sodium phosphate buffer (pH 5.0) containing 40–50% glycerol (see Note 1). If the compounds are not well soluble in buffer, first dissolve in a minimum amount of DMSO and then dilute with buffer containing 40–50% glycerol.

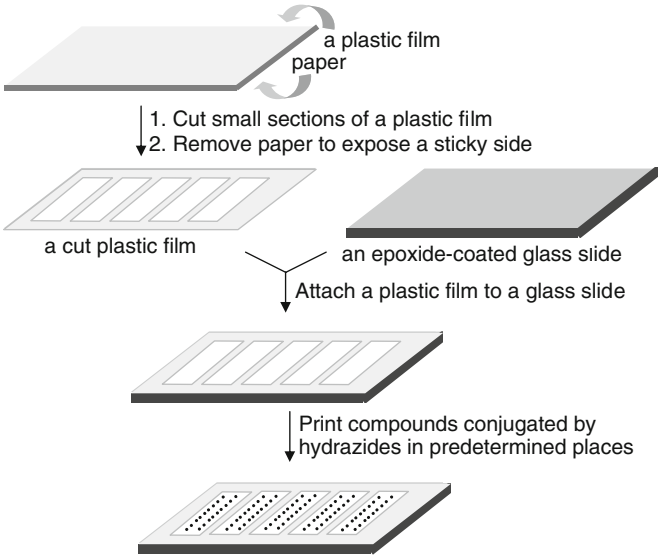


Fig. 3. Scheme for the attachment of a plastic film to modified glass slides.

5. Transfer 5–10 μl of the solution into a 384-well microplate. The V-shaped 384-well microplate is recommended since a small amount of solutions can be loaded into the microplate. Be careful not to form bubble during transferring the solutions. The microplate containing the solutions can be stored at –70°C for several months.
6. Cut parts of a plastic film (thickness: 0.1–0.2 mm) that is coated by adhesive at one side with a knife (see Fig. 3). Attach the blocked plastic film to an epoxide-coated glass slide prepared by the procedure in step 3 (see Note 6).
7. Print 1 nl of compound solutions from a 384-well microplate in predetermined places on epoxide-coated glass slides by using a robotic printing microarrayer (see Note 7). To prevent contamination of slides, the print chamber and printing pins should be clean.
8. After printing is completed, leave the slides in the print chamber (60% humidity) for 3 h at room temperature. Slides can be left overnight at room temperature in the print chamber.
9. After washing the slides with 30 ml of deionized water (5 min × 2), dry the slides by purging with Ar gas.
10. Drop 20–30 μl of a solution of 3% 2-aminoethanol or glycine in 10 mM sodium bicarbonate (pH 8.3) on each block of the slides by using a micropipette and incubate in the print chamber (60% humidity) for 0.5 h at room temperature. Unreacted epoxides on the slides are removed at this step (see Note 8).

11. After washing the slides with 30 ml of deionized water (5 min × 2), dry the slides by purging with Ar gas. Slides can be stored at room temperature in a desiccator for several weeks. However, for best results, prepare slides freshly prior to use.

3.2. Protein Labeling with Fluorescent Dyes

Proteins of choice are labeled with either fluorescein isothiocyanate (FITC, see Subheading 3.2.1) or Cy3/Cy5 (see Subheading 3.2.2). Cy3/Cy5-labeled proteins give usually better results for protein-binding studies than FITC-labeled ones because Cy3 and Cy5 dyes exhibit higher quantum yield and are more stable to light than fluorescein. However, Cy3-NHS and Cy5-NHS are much more expensive than FITC.

3.2.1. Labeling of Proteins by FITC

1. Prepare 0.1 ml of a solution of protein (~2 mg/1 ml) in 0.1 M sodium bicarbonate (pH 8.3). If protein activity is decreased by 0.1 M sodium bicarbonate, the labeling reaction can be carried out in 0.1 M sodium phosphate, HEPES or borate buffers at the same pH. However, do not use buffers containing free amines such as Tris or glycine since these buffers are reacted with FITC.
2. Add 2 µl of a solution of FITC (50 mg/ml in DMSO) to 0.1 ml of protein solution and mix thoroughly. Be careful not to foam the protein solution.
3. Incubate the reaction mixture with rocking for 0.5–1 h at room temperature. Keep the reaction in the dark since FITC is light-sensitive.
4. To remove unreacted dye, adjust the volume of the reaction mixture to 2.5 ml with protein storage buffer and load on the PD-10 desalting column that is prewashed with approximately 25 ml protein storage buffer (see Subheading 2.3).
5. Discard the flow-through. Unreacted dye can be alternatively removed from conjugated proteins by dialysis (see Note 9).
6. Elute with 3.5 ml protein storage buffer and collect the flow-through. Two colored bands are usually observed; the faster moving band contains the labeled protein and the slower band contains free dye.
7. Divide the solution into several portions and store at 4°C. The excitation and emission wavelengths of fluorescein are 492 and 520 nm, respectively. The fluorescence properties of a labeled protein and free dye are very similar. The labeled protein can be stored at 4°C for several weeks.

3.2.2. Labeling of Proteins by Cy3 or Cy5

1. Dissolve one tube of Cy3 or Cy5 monoreactive dye (GE Healthcare) in 30 µl DMSO and add 6 µl of the dye solution

to 400 μg protein in 200 μl of 0.1 M sodium bicarbonate (pH 8.3).

2. Mix thoroughly and incubate the mixture with rocking for 0.5–1 h at room temperature. Be careful not to foam the protein solution.
3. Adjust the volume of the reaction mixture to 2.5 ml with protein storage buffer and load on the PD-10 desalting column that is prewashed with approximately 25 ml protein storage buffer. Discard the flow-through.
4. Elute with 3.5 ml protein storage buffer and collect the flow-through. Two colored bands are usually observed; the faster moving band contains the labeled protein and the slower band contains free dye.
5. Divide the solution into several portions and store at 4°C. The excitation and emission wavelengths of Cy3 are 550 and 570 nm, respectively, and the excitation and emission wavelengths of Cy5 are 675 and 694 nm, respectively. The fluorescence properties of a labeled protein and free dye are very similar. The labeled protein can be stored at 4°C for several weeks.

3.3. Applications of Chemical Microarrays

3.3.1. Rapid Analysis of Protein Binding to Carbohydrates, Peptides, and Small Molecules

1. Before using the chemical microarrays, drop 20–30 μl of a solution of 10 mM PBS (pH 7.4) containing 1% BSA and 0.1% Tween 20 on each block of the slides prepared by the procedure in step 11 of Subheading 3.1 and incubate in the print chamber (humidity 60%) for 1–0.5 h at room temperature (see Note 10).
2. Rinse the slides with 10 mM PBS (pH 7.4) containing 0.1% Tween 20 to remove protein solution and wash with 30 ml of the same buffer with gentle shaking (10 min × 3). Rinse the slides with deionized water.
3. Dry the slides by purging with Ar gas and drop 20–30 μl of a solution of fluorescent dye-labeled proteins (1–10 μg/ml) in the appropriate buffer containing 0.1% Tween 20 on each block of the slides by using a micropipette (see Note 11). Handle proteins on ice at all times. This helps to preserve protein activity.
4. Incubate in the print chamber (60% humidity) for 0.5–1 h at room temperature.
5. Rinse the slides with protein buffer to remove unbound proteins. Remove the compartmentalized plastic film from glass slides and wash with 30 ml protein buffer containing 0.1% Tween 20 with gentle shaking (10 min × 3). Rinse the slides with deionized water.

6. Dry the protein-treated slides by purging with Ar gas. The slides probed with fluorescent dye-labeled proteins can be stored at room temperature in a desiccator for several weeks.
7. Scan slides using a microarray scanner fitted with the appropriate filters. Keep slides clean at all times. When slides are contaminated by dust, they will display high fluorescence intensity and consequently the fluorescence intensity of the desired spots will be unclear. Process fluorescence data using ImaGene 6.1 software and analyze data using Origin Pro 7.0 or other software (see Fig. 4).

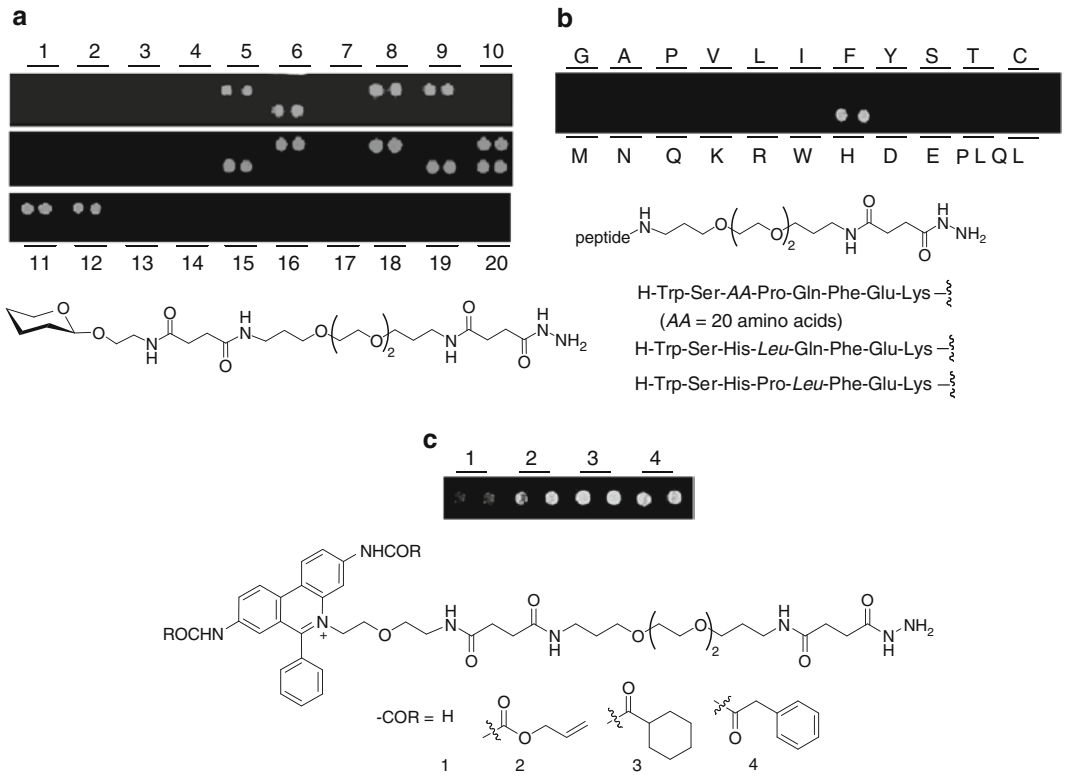


Fig. 4. Rapid analysis of protein binding to carbohydrates, peptides, and small molecules using chemical microarrays. (**a**) Carbohydrate-binding patterns of proteins. Carbohydrate microarrays were probed with Cy3-labeled *Wheat germ* agglutinin (WGA) (*top*), FITC-labeled *Concanavalin* A (ConA) (*middle*), and Cy5-labeled *Aleuria aurantia* lectin (AA) (*bottom*) (1: Fuc-α, 2: Fuc-β, 3: Gal-α, 4: Gal-β, 5: GalNAc-α, 6: Glc-α, 7: Glc-β, 8: GlcNAc-α, 9: GlcNAc-β, 10: Man-α, 11: Xyl-α, 12: Xyl-β, 13: Glcβ1,4Glc-β, 14: Galβ1,4Glc-β, 15: Glcα1,4Glc-β, 16: Galβ1,4GlcNAc-β, 17: Galβ1,6Man-α, 18: Glcβ1,6Man-α, 19: Manα1,6Man-α, 20: Manα1,6Manα1,6Man-α). (**b**) peptide-binding patterns of streptavidin (single letter amino acid codes: 20 amino acids, P → L; replacement of P in peptide by L, Q → L; replacement of Q in peptide by L). Peptide microarrays were treated with Cy3-streptavidin. (**c**) Small molecule-binding patterns of acetylcholinesterase (AChE). Small molecule microarrays were incubated with Cy5-acetylcholinesterase.

3.3.2. Profiling of β-1,4-Galactosyltransferase Activity

1. Drop a 20–30 μl solution of β-1,4-galactosyltransferase (1 mU), $MnCl_2$ (10 mM) and UDP-Gal (1 mM) in HEPES buffer (50 mM, pH 7.5) on each block of the BSA-pretreated slides containing various carbohydrates by using a micropipette.
2. Incubate the slides in the print chamber (80% humidity) at 37°C for 3 h.
3. Rinse the slides with HEPES buffer to remove enzyme solutions and wash with 30 ml PBS buffer (pH 7.4) containing 0.1% Tween 20 with gentle shaking (10 min × 3). Further wash the slides with 30 ml of 35 mM SDS in PBS buffer (pH 7.4) under sonication at 60–65°C for 5 min (see Note 12). Rinse the slides with deionized water.
4. Dry the slides by purging with Ar gas.
5. Drop a 20–30 μl solution of Cy3-*Ricinus communis* agglutinin I (RCA_{120}) that binds to galactose-containing glycans, in PBS (pH 7.4) containing 0.1% Tween 20 on each block of the slides by using a micropipette. Incubate the slides in the print chamber (60% humidity) for 0.5–1 h at room temperature.
6. Rinse the slides with PBS (pH 7.4) to remove protein solutions. Remove the compartmentalized plastic film from glass slides and wash with 30 ml PBS (pH 7.4) containing 0.1% Tween 20 with gentle shaking (10 min × 3). Rinse the slides with deionized water.
7. Dry the slides by purging with Ar gas. The slides can be stored in a desiccator at room temperature for several weeks.
8. Scan slides using a microarray scanner fitted with the appropriate filters. Process fluorescence data using ImaGene 6.1 software and analyze data using Origin Pro 7.0 or other software (see Fig. 5).

3.3.3. Determination of Dissociation Constants (K_d Values) for Protein–Carbohydrate Interactions

1. Prepare 5–10 μl of hydrazide-conjugated LacNAc-α and -β solutions in 0.1 M sodium phosphate buffer (pH 5.0) containing 40–50% glycerol.
2. Print 1 nl of the solutions from a 384-well microplate in predetermined places on epoxide-coated glass slides by using a robotic printing microarrayer.
3. After printing is completed, leave the slides in the print chamber (60% humidity) for 3 h at room temperature.
4. After washing the slides with 30 ml of deionized water (3 min × 3), dry the slides by purging with Ar gas.
5. Drop 20–30 μl of a solution of 3% 2-aminoethanol in 10 mM sodium bicarbonate (pH 8.3) on each block of the slides by using a micropipette and incubate in the print chamber

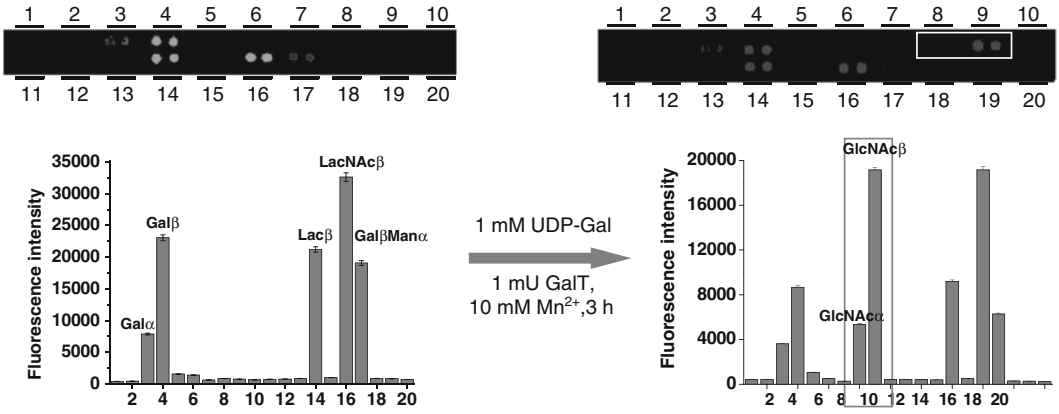


Fig. 5. Profiling of β-1,4-galactosyltranserase activity with microarrays containing twenty carbohydrates. Carbohydrate microarrays were incubated with 1 mU β-1,4-GalT in the presence of 10 mM $MnCl_2$ and 1 mM UDP-Gal for 3 h at 37°C and then probed with Cy3-RCA_{120}. Fluorescence images of RCA_{120}-treated slides before (*left*) and after (*right*) treatment with enzyme (immobilized glycans on the surfaces are the same as those in Fig. 4a). Only GlcNAc-α and -β were converted to LacNAc-α and -β, respectively, by this enzyme. Data are the average ± S.D. of triplicate determinants.

(60% humidity) for 0.5 h at room temperature to quench unreacted epoxides.

6. After washing the slides with 30 ml of deionized water (3 min × 3), dry the slides by purging with Ar gas.
7. Drop 20–30 μl of a solution of 1% BSA and 0.1% Tween 20 in 10 mM sodium bicarbonate (pH 8.3) on each block of the slides by using a micropipette and incubate in the print chamber (humidity 60%) for 0.5 h at room temperature.
8. Rinse the slides with 10 mM PBS (pH 7.4) containing 0.1% Tween 20 to remove protein solution and wash with 30 ml of the same buffer with gentle shaking (10 min × 3). Rinse the slides with deionized water. Dry the slides by purging with Ar gas.
9. Drop 20–30 μl of a solution of 0.1 nM to 1 mM concentrations of Cy3-RCA_{120} (20 μg/ml) in the 10 mM PBS (pH 7.4) containing 0.1% Tween 20 on each block of the slides by using a micropipette.
10. Incubate in the print chamber (60% humidity) for 1 h at room temperature.
11. Rinse the slides with 10 mM PBS (pH 7.4) containing 0.1% Tween 20 to remove protein solution and wash with 30 ml wash buffer with gentle shaking (3 min × 3). Rinse the slides with deionized water.
12. Scan slides using a microarray scanner fitted with the appropriate filters. Process fluorescence data using Imagene 6.1 software, and analyze data using Origin Pro 7.0 (see Fig. 6).

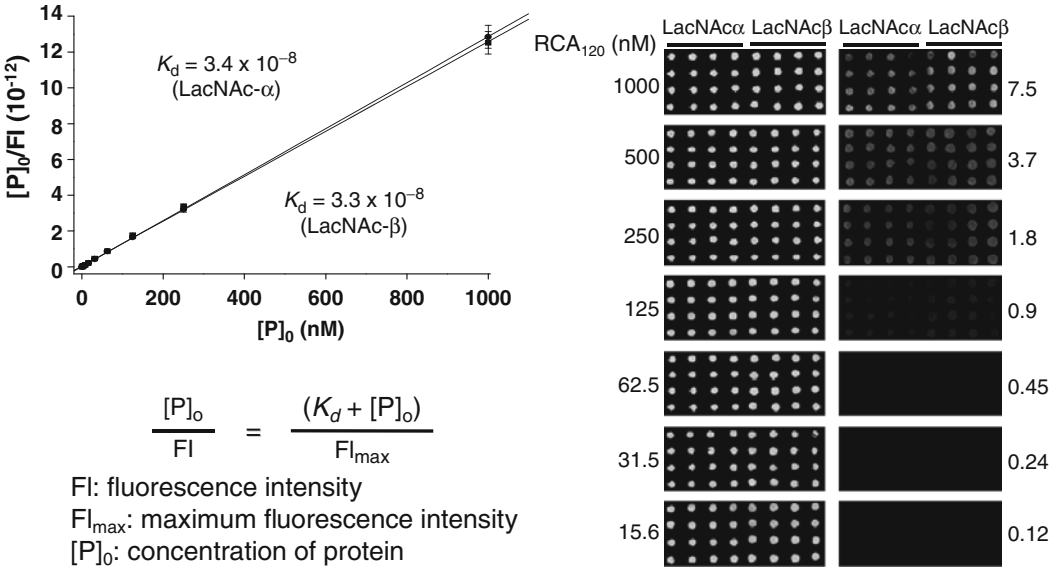


Fig. 6. Determination of dissociation constants (K_d) for RCA_{120}-surface-linked LacNAc interactions using carbohydrate microarrays (*filled circle*: LacNAc-α, *filled square*: LacNAc-β, *FI* fluorescence intensity) (K_d values were determined by using equation $(P)_o/FI = K_d/FI_{max} + (P)_o/FI_{max}$) (17, 19).

4. Notes

1. Addition of glycerol to sample solutions suppresses the unwanted evaporation of nanodroplets during spotting and immobilization. Evaporation of solutions results in uneven, inefficient immobilization of compounds.
2. Amine-coated glass slides can be prepared by a known procedure (20). However, it is recommended that nonexperts should purchase the derivatized glass slides from commercial suppliers since the quality of the modified glass slides is of great importance for reproducible results.
3. High concentrations of proteins sometimes result in the precipitation of proteins. In this case, the concentration of proteins is decreased.
4. Although shaking can be omitted during modification of slide surfaces, gentle stirring on an orbital shaker is recommended to increase the efficiency of glass surface modification.
5. Argon purging is preferred to centrifugation as a drying method since the former can remove the contaminating dust on the slides.

6. Use of the blocked plastic film facilitates compartmentalization that is required for simultaneously incubation of a single slide with several proteins.
7. The humidity in the print chamber should be kept at 60% to avoid the unwanted evaporation of nanodroplets during spotting.
8. Quenching of the unreacted epoxide groups on the surface by the treatment with glycine or 2-aminoethanol can be omitted. Incubation of the chemical microarrays with BSA prior to probing with proteins quenches the unreacted epoxide groups.
9. Whereas dialysis gives a higher concentration of conjugated protein than gel filtration, the former method normally takes more time (1 day) than the latter one (within 1 h). Therefore, it is recommended that gel filtration should be performed for the rapid removal of free dye.
10. One of the biggest problems for microarray experiments is the nonspecific interaction of the probing proteins with derivatized surfaces. Treatment of the microarrays with BSA considerably decreases these nonspecific interactions. Hydrophilically modified surfaces such as poly(ethylene glycol) (PEG) considerably suppress these undesired interactions even without the treatment with BSA.
11. Tween 20 should be added to a solution of dye-labeled proteins prior to use since Tween 20 in protein solutions results in the decrease of protein activity during storage.
12. Omission of sonication of the enzyme-treated slides in a hot SDS solution results in a high background fluorescence.

Acknowledgments

This work was supported by grants of the National Creative Research Initiative and WCU programs (KOSEF/MEST).

References

1. Koch, W. H. (2004) Technology platforms for pharmacogenomic diagnostic assays. *Nat Rev Drug Discov* **3**, 749–61.
2. Villeneuve, D. J., and Parissenti, A. M. (2004) The use of DNA microarrays to investigate the pharmacogenomics of drug response in living systems. *Curr Top Med Chem* **4**, 1329–45.
3. Zhu, H., and Snyder, M. (2003) Protein chip technology. *Curr Opin Chem Biol* **7**, 55–63.
4. Wilson, D. S., and Nock, S. (2003) Recent developments in protein microarray technology. *Angew Chem Int Ed* **42**, 494–500.
5. Uttamchandani, M., and Yao, S. Q. (2008) Peptide microarrays: next generation biochips for detection, diagnostics and high-throughput screening. *Curr Pharm Des* **14**, 2428–38.
6. Park, S., Lee, M.-R., and Shin, I. (2008) Carbohydrate microarrays as powerful tools in

studies of carbohydrate-mediated biological processes. *Chem Commun* **37**, 4389–99.

7. Shin, I., Tae, J., and Park, S. (2007) Carbohydrate microarray technology for functional glycomics. *Curr Chem Biol* **1**, 187–99.
8. Shin, I., Park, S., and Lee, M.-R. (2005) Carbohydrate microarrays: an advanced technology for functional studies of glycans. *Chem Eur J* **11**, 2984–901.
9. Lee, M.-R., and Shin, I. (2005) Facile preparation of carbohydrate microarrays by site-specific, covalent immobilization of unmodified carbohydrates on hydrazide-coated glass slides. *Org Lett* **7**, 4269–72.
10. Park, S., Lee, M. R., Pyo, S. J., and Shin, I. (2004) Carbohydrate chips for studying high-throughput carbohydrate-protein interactions. *J Am Chem Soc* **126**, 4812–9.
11. Park, S., and Shin, I. (2002) Fabrication of carbohydrate chips for studying protein-carbohydrate interactions. *Angew Chem Int Ed* **41**, 3180–2.
12. Park, S., Lee, M.-R., and Shin, I. (2009) Construction of carbohydrate microarrays by using one-step, direct immobilizations of diverse unmodified glycans on solid surfaces. *Bioconjugate Chem* **20**, 155–62.
13. Vegas, A. J., Fuller, J. H., and Koehler, A. N. (2008) Small-molecule microarrays as tools in ligand discovery. *Chem Soc Rev* **37**, 1385–94.
14. Uttamchandani, M., Walsh, D. P., Yao, S. Q., and Chang, Y. T. (2005) Small molecule microarrays: recent advances and applications. *Curr Opin Chem Biol* **9**, 4–13.
15. Kuruvilla, F. G., Shamji, A. F., Sternson, S. M., Hergenrother, P. J., and Schreiber, S. L. (2002) Dissecting glucose signalling with diversity-oriented synthesis and small-molecule microarrays. *Nature* **416**, 653–7.
16. Lee, M. R., and Shin, I. (2005) Fabrication of chemical microarrays by efficient immobilization of hydrazide-linked substances on epoxide-coated glass surfaces. *Angew Chem Int Ed* **44**, 2881–4.
17. Park, S., and Shin, I. (2007) Carbohydrate microarrays for assaying galactosyltransferase activity. *Org Lett* **9**, 1675–8.
18. Park, S., Lee, M.-R., and Shin, I. (2007) Fabrication of carbohydrate chips and their use to probe protein-carbohydrate interactions. *Nat Protoc* **2**, 2747–58.
19. Liang, P.-H., Wang, S.-K., and Wong, C.-H. (2007) Quantitative analysis of carbohydrate-protein interactions using glycan microarrays: determination of surface and solution dissociation constants. *J Am Chem Soc* **128**, 11177–84.
20. Moon, J. H., Shin, J. W., Kim, S. Y., and Park, J. W. (1996) Formation of uniform aminosilane thin layer: an imine formation to measure relative surface density of the amino group. *Langmuir* **12**, 4621–4.

Chapter 17

A Microarray-Based Method to Perform Nucleic Acid Selections

Olga Aminova and Matthew D. Disney

Abstract

This method describes a microarray-based platform to perform nucleic acid selections. Chemical ligands to which a nucleic acid binder is desired are immobilized onto an agarose microarray surface; the array is then incubated with an RNA library. Bound RNA library members are harvested directly from the array surface via gel excision at the position on the array where a ligand was immobilized. The RNA is then amplified via RT-PCR, cloned, and sequenced. This method has the following advantages over traditional resin-based Systematic Evolution of Ligands by Exponential Enrichment (SELEX): (1) multiple selections can be completed in parallel on a single microarray surface; (2) kinetic biases in the selections are mitigated since all RNA binders are harvested from an array via gel excision; (3) the amount of chemical ligand needed to perform a selection is minimized; (4) selections do not require expensive resins or equipment; and (5) the matrix used for selections is inexpensive and easy to prepare. Although this protocol was demonstrated for RNA selections, it should be applicable for any nucleic acid selection.

Key words: Nucleic acids, SELEX, Microarrays, Screening, RNA motifs, Internal loops

1. Introduction

Nucleic acids that are selected to bind to a small molecule (1–3), protein (4–7), or whole cells (8) have a variety of applications that range from diagnosing (9, 10) and treating disease (11, 12) to deciphering the origins of life (13, 14). Many methods have been developed to select nucleic acids that bind a small molecule of interest; Systematic Evolution of Ligands by Exponential Enrichment (SELEX) is most frequently used. All of these methods require the isolation of active nucleic acids from inactive ones. These separations are most commonly completed by using affinity chromatography in which a ligand is conjugated onto a resin (4).

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4_17, © Springer Science+Business Media, LLC 2010

Capillary electrophoresis (CE) (15) has also been used to perform selections and does not require immobilization of a ligand onto a matrix. CE requires, however, that the mobility of the bound nucleic acid be different from that of the unbound. Though powerful, both methods have disadvantages. For example, in resin-based selections, kinetic biases can be introduced because a high concentration of the bound ligand is washed over a resin to elute the binders (16). This, the resin-based selections, can select against the highest affinity binders since they are the most difficult to compete off. CE SELEX is not affected by kinetic biases but does require specialized equipment. Neither method allows for multiple selections to be completed in parallel.

In an effort to complete multiple selections in parallel, a microarray-based method was developed. Microarrays are advantageous platforms for screening and performing selections because of the small amounts of ligand and analyte required, and the ability of microarrays to probe thousands of interactions in parallel. These advantages are best illustrated in the widespread uses of microarrays, which include studying gene expression (17), and protein–protein (18), carbohydrate–protein (19), cell–ligand (20, 21), and small molecule–protein interactions (22–24). In order to develop a microarray-based method to complete selections, a microarray surface that is robust enough for ligand screening and allows bound RNAs to be harvested directly from the array surface is required. We found that the optimal surface is an agarose-coated microarray (25, 26). Bound RNAs can be harvested from the array surface by simple excision of the agarose from ligand-functionalized positions (27, 28). Additionally, agarose provides an inexpensive and three dimensional surface for high ligand loading. It can also be functionalized to provide a chemical handle for immobilization of a variety of reactive groups. Most importantly, by harvesting all ligand-bound RNAs via gel excision, kinetic biases found in resin-based selections may be mitigated (27).

Not only does completing multiple selections in parallel on a microarray surface increase throughput, but it also allows for the identification of the highest affinity and specific RNA-ligand interactions. Higher affinity RNA-ligand interactions are selected at lower ligand loadings that captured the members of an RNA library (27). In contrast, separate experiments would be required for each ligand loading to identify higher affinity interactions using resin-based SELEX. Specific RNA-ligand interactions are identified using the microarray selection procedure because it may set up a competition experiment between the arrayed ligands for the members of the RNA library (28).

In this protocol, the steps for completion of a microarray-based selection are described (Fig. 1). We specifically focus on ligands that are immobilized onto an agarose array surface using

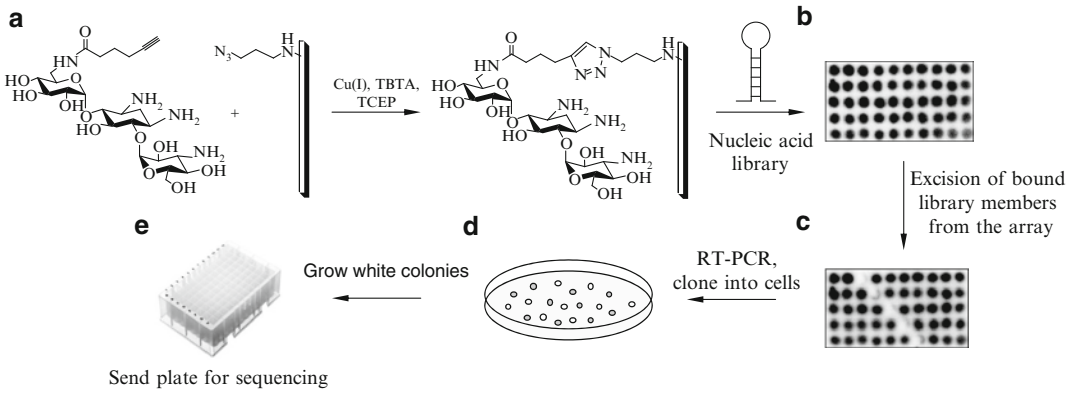


Fig. 1. Schematic of the microarray-based selection process: (**a**) immobilization of small molecules on an activated agarose microarray via a Huisgen 1,3-dipolar cycloaddition reaction. (**b**) An image of the slide after hybridization with radiolabeled RNA. (**c**) An image of the same slide after the mechanical removal of bound RNA. (**d**) A schematic of an agar plate after transformation with a vector containing selected members of the library. (**e**) Colonies that are *white* from *blue/white* screening should be grown in culture and submitted for sequencing to deconvolute the selected members of the library.

a Huisgen cycloaddition reaction in which the array displays either azide or alkyne groups (29, 30), however, other immobilization chemistries are compatible with this platform, including immobilization of amine-containing ligands via reductive amination (25). This approach should be applicable to any nucleic acid-based selection.

2. Materials

2.1. Functionalization of Agarose Slides

1. 31.8 mM solution of $NaCNBH_3$: Dissolve 0.2 g of sodium cyanoborohydride in 80 ml 1× PBS and 20 ml ethanol. Prior to applying this solution to the array surface (preferred vendor is Sigma Aldrich, Silane Prep Slides), ensure that all of the $NaCNBH_3$is dissolved by stirring the solution for 2–3 min. It is important to prepare this solution fresh every time.
2. 10× phosphate buffered saline (PBS): Dissolve 14.2 g of Na_2HPO_4, 2.45 g of KH_2PO_4, 81.8 g of NaCl, 1.86 g of KCl in 900 ml of nanopure water. Adjust the pH to 7.5 using NaOH or H_3PO_4. Add nanopure water to bring volume to 1 L. Store the solution at room temperature.
3. 0.2% Sodium dodecyl sulfate (SDS): Dissolve 2 g of SDS in 900 ml of nanopure water; add water to make 1 L of solution. Store the solution at room temperature.

2.2. Spotting of Small Molecules onto Microarrays

1. 10× phosphate buffer: Prepare the following solutions:

   1 M K_2HPO_4 (dissolve 1.74 g of K_2HPO_4 in 10 ml of nanopure water).

   1 M KH_2PO_4 (dissolve 1.36 g of KH_2PO_4 in 10 ml of nanopure water).

   1 M Na_2HPO_4 (dissolve 1.42 g of Na_2HPO_4 in 10 ml of nanopure water).

   1 M NaH_2PO_4 (dissolve 1.2 g of NaH_2PO_4 in 10 ml of nanopure water).

   Prepare a 100 mM potassium phosphate solution by mixing 940 µl of 1 M K_2HPO_4, 60 µl of 1 M KH_2PO_4, and 9 ml of nanopure water.

   Prepare a 100 mM sodium phosphate solution by mixing 932 µl of 1 M Na_2HPO_4, 68 µl of 1 M NaH_2PO_4, and 9 ml of nanopure water.

   Finally, mix equal volumes of the two 100 mM solutions together; this solution should have a pH of ~8. Store the solution at room temperature.
2. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA) solution: Stock solutions of TBTA should be made in 4:1 mixture of 2-butanol:DMSO. Store at 4°C.
3. 10% ethylene glycol solution: Mix 20 ml of ethylene glycol with 180 ml of nanopure water. Store the solution at room temperature.

2.3. Hybridization of RNA onto the Slides

1. 10× Hybridization Buffer: Dissolve 11.36 g of Na_2HPO_4, 3.72 g of $Na_2EDTA{\cdot}2H_2O$, 105.2 g of NaCl in 900 ml of nanopure water; adjust the pH to 7.0 with HCl. Add nanopure water to bring volume to 1 L. Store the solution at room temperature.
2. DEPC-treated water: Add 100 µl of Diethylpyrocarbonate (DEPC) to 1 L of distilled water. Mix well and incubate at room temperature for 2 h. Autoclave on the liquid cycle for 20 min at 121°C to inactivate the DEPC. Store the water at room temperature.

2.4. Cell Cloning

1. LB medium: Dissolve 10 g of bacto tryptone, 5 g of bacto yeast extract, 10 g of NaCl, and 1 ml of 1 M NaOH in 900 ml of de-ionized water. Adjust the pH to 7.0 with 1 M NaOH. Sterilize by autoclaving on the liquid cycle at 121°C for 20 min. Store the solution at room temperature.
2. Terrific broth medium Dissolve 12 g of tryptone, 24 g of yeast extract, and 4 ml of glycerol in 900 ml deionized water. Sterilize by autoclaving on the liquid cycle at 121°C for 20 min. Add 100 ml of 0.17 M KH_2PO_4 + 0.72 M K_2HPO_4

that has been sterile filtered. Store the solution at room temperature.

3. SOC medium Dissolve 20 g of bacto tryptone, 5 g of bacto yeast extract, 0.5 g of NaCl, 10 ml of 250 mM KCl, and 3.6 g of glucose in 900 ml of water. Adjust the pH to 7.0 using 1 M NaOH. Sterilize by autoclaving at 121°C for 20 min on the liquid cycle. Store the solution at room temperature.
4. 100 mM Isopropyl-β-D-thiogalactoside (IPTG): Dissolve 238 mg of IPTG in 10 ml of nanopure water. Store the solution in a brown glass bottle or wrap the container in aluminum foil as IPTG is light sensitive. Store at –20°C.
5. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal): Dissolve 200 mg of X-gal in 10 ml of DMF. Store the solution in a brown glass bottle or wrap the container in aluminum foil as X-gal is light sensitive. Store at –20°C.

2.5. Polymerase Chain Reaction and RNA Transcription

1. 10× PCR buffer: Dissolve the following in 45 ml nanopure water: 1.86 g of KCl, 0.6 g of Tris base, 500 µl of Triton X-100. Use KOH to adjust the pH of the solution to 9.0. Bring the total volume to 50 ml. Store the solution at –20°C. A suitable buffer supplied by the manufacturer can also be used.
2. To transcribe RNA, we use RNAMaxx transcription kit (Stratagene).

2.6. Polyacrylamide Gel Electrophoresis

1. 10× TBE Buffer: Dissolve 108 g of Tris base, 55 g of boric acid, 80 ml of 0.5 M EDTA in 850 ml of nanopure water. Adjust the pH to 8.3 using HCl. Bring volume to 1 L. Store the solution at room temperature.
2. 0.5 M EDTA: Dissolve 14.9 g of EDTA in 900 ml of nanopure water. Adjust the pH to 9.0 with NaOH. Bring volume to 1 L with nanopure water. The solution is stored at room temperature.
3. 2× Loading Buffer: Dissolve 24 g of urea, 0.372 g of $Na_2EDTA{\cdot}2H_2O$, and 0.0121 g of Tris base in approximately 30 ml of nanopure water. Bring volume to 50 ml with water. Check the pH of solution; it should be approximately 7.5. Do not try to adjust the pH, as this will result in the introduction of excess of salt which will cause problems during gel electrophoresis. Add 5 mg of Orange G dye and store the solution at room temperature.
4. 2× Loading Buffer with bromophenol blue and xylene cyanol: Make 2× Loading buffer as described above, but do not add Orange G. Instead, add 2 mg of bromophenol blue and 2 mg of xylene cyanol. Store at room temperature.

3. Methods

3.1. Preparation of Agarose Slides

1. Prepare 1% agarose solution (w/v) using nanopure water. Melt in a microwave on high for 2–3 min, swirling the solution every 20–30 s.
2. While the solution is hot, apply ~1.5 ml to the surface of a glass slides using a P-1000 pipette. Ensure that the solution is spread evenly over the slide surface. Allow the agarose to dry to a thin film overnight (Fig. 2a, b).

3.2. Functionalization of Agarose Slides

1. Prepare a solution of 0.02 M $NaIO_4$ in water. Submerge the slides in this solution for 30 min at room temperature, and then wash with nanopure water for 30 min.
2. Submerge the slides in 10% (v/v) ethylene glycol for 1 h at room temperature to quench residual $NaIO_4$. Wash with water for 1.5 h, changing water every 20 min.
3. (A) To prepare microarrays of amine-displaying ligands, complete the following:
   1. Allow the slides to dry.
   2. Prepare spotting solutions as follows: small molecule at desired concentration (typically serially diluted from 5 mM to 1 μM), 0.1 M $NaHCO_3$, and 10% glycerol. Spot 0.4 μl of the solutions onto aldehyde-agarose slides in duplicate.

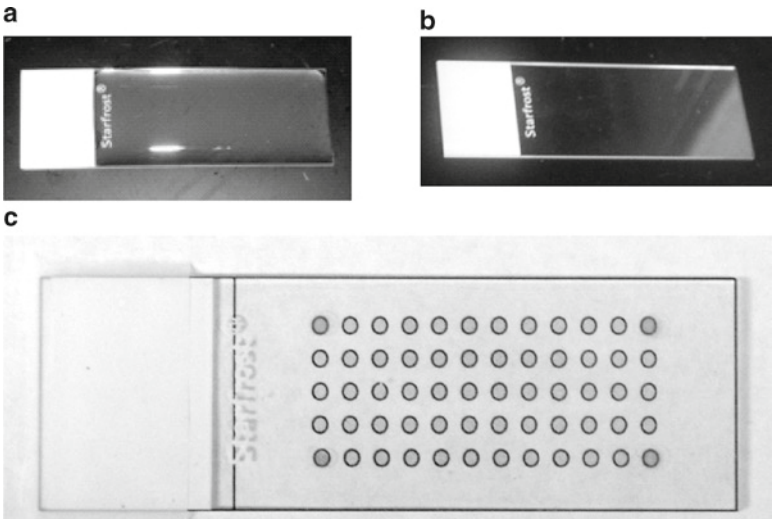


Fig. 2. Images of agarose arrays during the preparation and selection of bound members. (**a**) An agarose slide that has solidified to a gel. (**b**) An agarose-coated microarray that has dried to a clear film. (**c**) An image of an agarose array that is placed on top of a paper grid to image use as a guide for spotting ligands; the *spots to indicate the four corners of the grid* have been put on the array using a hydrophobic marker and help align the grid for precise excision of bound nucleic acid on an array. The same grid is used to align the image of slides after hybridization with an RNA library.

3. Incubate for 3 h at room temperature in a humidity chamber (box containing a saturated solution of NaCl). Wash the slides 3 × 10 min with 1× Hybridization Buffer, followed by water, 2 × 10 min. Continue to step 6.
   (B) For microarrays of alkyne-displaying ligands, submerge the slides in a solution of 0.1 M $NaHCO_3$ (pH 8.5) and 10 mM 3-azidopropylamine for 3 h at room temperature. Continue to step 6.
   (C) For microarrays of azide-displaying ligands, replace 10 mM 3-azidopropylamine with 10 mM propargylamine. Continue to step 6.
4. Submerge slides in solution of 31.8 mM $NaBH_3$ for 3 min to reduce the imine formed on the microarray surface.
5. Wash the slides with 0.2% SDS, 3 × 15 min, and then with water, 2 × 15 min.
6. Dry the slides under a stream of air. *(This is the last step for microarrays of amine-displaying ligands.)*

3.3. Immobilization of Aminoglycosides on the Slide Surface

1. Prepare spotting solutions as follows:
   (a) For azide-displaying small molecules: the small molecule at the desired concentration (typically two to threefold serially diluted from 5 mM to 1 μM), 10 mM Tris-HCl, pH 8.5, 1 mM $CuSO_4$, 100 μM ascorbic acid, 100 μM TBTA (29), and 10% glycerol. Spot 0.4 μl of the solutions onto alkyne-agarose slides in duplicate.
   (b) For alkyne-displaying small molecules: the small molecule at the desired concentration (typically serially diluted from 5 mM to 1 μM), 1× phosphate buffer, 1 mM $CuSO_4$, 0.5 mM TCEP, 100 μM TBTA (29), and 10% glycerol. Spot 0.4 μl of the solutions onto azide-agarose slides in duplicate.
2. Incubate for 3 h at room temperature in a humidity chamber (box containing a saturated solution of NaCl). Wash the slides 3 × 10 min with 1× Hybridization Buffer, followed by water, 2 × 10 min.

3.4. Transcription of RNA Using DNA Template

1. Amplify the randomized DNA template that encodes the RNA library by PCR using a forward primer that includes a T7 RNA polymerase promoter. Set up the reaction as follows: 1× PCR buffer, 0.3 mM dNTPs, 4.25 mM $MgCl_2$, 2 μM forward primer, 2 μM reverse primer, 20 nM template, and one unit of Taq DNA polymerase in a total volume of 50 μl. Alternatively, anneal two strands of complementary DNAs encoding an RNA polymerase promoter and the desired RNA library.

2. While PCR is running, pour an agarose gel containing ethidium bromide to analyze the PCR reaction. Choose the percentage of agarose that is appropriate for the size of the PCR product. For the RNAs used herein, 2% agarose is sufficient.
3. Mix 3 μl of PCR product with 1 μl 50% glycerol. Load the PCR product and an aliquot of a 100 bp DNA ladder into separate wells of the agarose gel; run the gel at 100 V for 15–30 min, or until proper separation is obtained. Visualize the DNA using a transilluminator or handheld UV lamp. Keep this gel to check the transcription reaction.
4. Transcribe the PCR product using any transcription protocol that utilizes an RNA polymerase (31). Manufacturers usually provide procedures for transcription of unlabeled RNA or ^{32}P-internally labeled RNA; add 2 μL of α-^{32}P ATP, or 20 μCi, to each transcription reaction.
5. While the RNA is transcribing, pour a 16 cm × 19.7 cm × 0.8 mm denaturing polyacrylamide gel to purify the RNA transcript. The percentage of acrylamide used should be appropriate for the size of the RNA library. For the library described here, 12% acrylamide gels are used (32).
6. Check that the transcription reaction was successful by agarose gel. Pour a 2% agarose gel and mix about 100 ml of 1× TBE buffer with 5 μl of 10 mg/ml aqueous ethidium bromide solution. Mix 0.5 μl of the transcription reaction with 5 μl of 15% glycerol. Load the sample and an aliquot of a 100 bp DNA ladder on an agarose gel. Run the gel at 100 V for 15–30 min or until proper separation is obtained. Visualize the DNA using a transilluminator or handheld UV lamp.
7. Once the transcription reaction is complete, add 1 unit of RNase-free DNase and incubate the sample for 30 min at 37°C.
8. Add an equal volume of 2× Loading Buffer to the transcription reaction. Load on the acrylamide gel prepared in step 5. Also, load running dyes (bromophenol blue and xylene cyanol in 1× Loading Buffer) to determine when proper separation of the RNA has been obtained. Run the gel at 300 V for 1–3 h using the running dyes as a guide.
9. Visualize the RNA transcript:
   (a) If the RNA is transcribed without radioactivity, identify the transcript by UV shadowing. Place a TLC plate with a fluorescent indicator under the gel, and excise the product band with a razor blade. (The product will appear purple.)
   (b) If the RNA is transcribed in the presence of α-[^{32}P] ATP, the product can be identified by exposing the gel

to a phosphorimager screen. Wrap the gel in saran wrap before placing the screen on top of the gel. Use the image as a template to excise the product band with a razor blade.

10. Place the gel slice into a 15 ml conical tube. Add 3 ml of 0.3 M NaCl in DEPC-treated water and tumble at 4°C overnight.
11. Centrifuge the sample to pellet the gel pieces, and transfer the supernatant to a fresh 15 ml conical tube. Use 2-butanol to concentrate the RNA to approximately 0.5 ml, and ethanol precipitate by adding 2.5 volumes of ethanol to the sample, and then place the sample at –20°C for at least 15 min. Pellet the RNA by spinning in a centrifuge for 10 min at 10,000 × *g* at 4°C (32).
12. Resuspend the RNA in 100 µl DEPC-treated water. Determine the concentration of RNA using UV absorption at 260 nm and the corresponding extinction coefficient (for example, obtained from HyTher server, HyTher version 1.0, Nicolas Peyret, and John SantaLucia, Jr., Wayne State University) (33, 34). Since this is an RNA library, the extinction coefficient is an estimate.

3.5. 5′-End Labeling of RNA (If RNA Is Not Internally Labeled)

1. Remove 5′ phosphate group by incubating 20 pmol RNA, 1× alkaline phosphatase buffer, and three units of calf intestinal alkaline phosphatase (CIAP) in 100 µl total volume at 37°C for 1 h.
2. Remove CIAP from the reaction by phenol/chloroform/isoamyl alcohol extraction and then ethanol precipitate the RNA with 10 µg of glycogen. Place the sample in a vacuum concentrator for 1–2 min to remove residual ethanol.
3. Kinase the RNA by dissolving the above pellet in 1× kinase buffer containing 3 µl [γ-^{32}P] ATP and five units of T4 polynucleotide kinase. Incubate the mixture at 37°C for 1 h. Mix with an equal volume of 2× Loading Buffer. Purify the reaction by denaturing polyacrylamide gel electrophoresis (PAGE) and extract RNA as described in Subheading 3.4, step 10.

3.6. Hybridization of RNA to the Slide

1. Prepare a solution of ^{32}P-labeled RNA in 1× Hybridization Buffer. We generally use the amount of RNA that gives ≥2,000 cpm by Geiger counter for every slide. Refold the RNA as appropriate. (For example, heat the sample at 60°C for 5 min and allow to slowly cool to room temperature on bench top.) If the isolation of specific RNA-ligand interactions in which the RNAs are small secondary structures is desired, then competitors – oligonucleotide mimics of the constant regions in the library – should be added to the

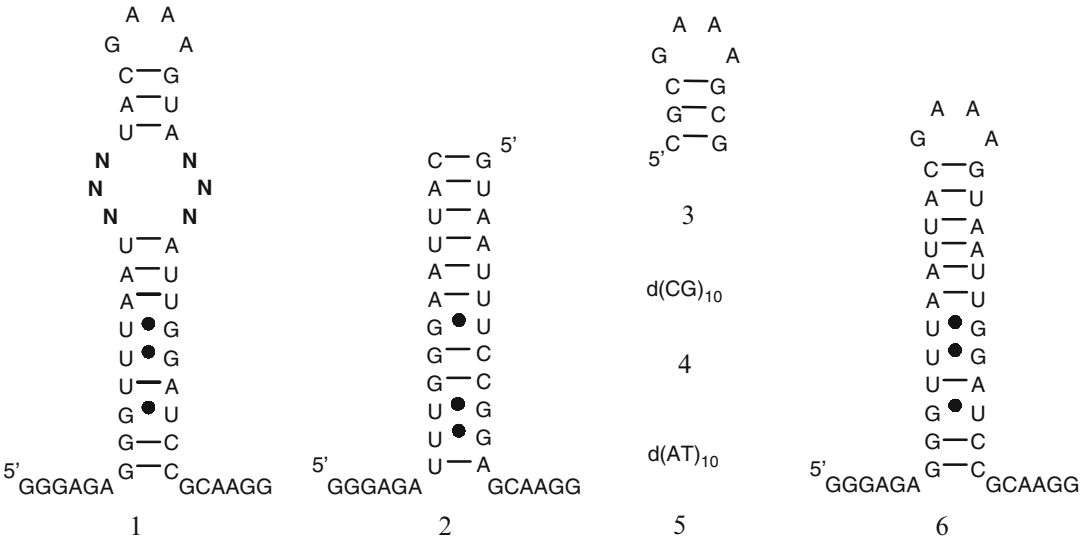


Fig. 3. An example of oligonucleotides that may be used to identify RNA internal loop motifs that bind ligands. Oligonucleotide **1** is an internal loop library with six randomized positions (N) that contains 4,096 unique members; **2–5** are examples of competitors used to ensure that the interaction between a small molecule and RNA is specific; and **6** is the cassette in which the internal loop library is embedded.

hybridization solution (Fig. 3). If only the isolation of aptamers that bind a ligand is desired, then it is not necessary to use competitor oligonucleotides. Add 1× Hybridization Buffer and competitor oligonucleotides if appropriate to a total volume of 500 µl.

2. While the RNA is cooling to room temperature, prehybridize ligand-functionalized slides with 500 µl of 0.1% BSA (w/v) in 1× Hybridization Buffer for 5 min. Distribute the buffer evenly over the surface of the slide by placing a piece of parafilm over the applied solution. After prehybridization, remove parafilm and shake off the buffer.
3. Apply RNA solution to the slide, distributing it evenly over the surface using custom-cut 1 in.×3 in. piece of parafilm. Incubate at room temperature for at least 20 min.
4. Wash the slides with 50 ml of 1× Hybridization Buffer with slight agitation 3 × 10 min. Gently blow air over the slide for 1 min to remove excess of buffer, and dry at room temperature by sitting on bench. Wrap the slide in saran wrap and expose to a phosphorimager screen at –20°C. Exposure time depends on how radioactive the slides are (3 h – overnight).
5. Scan the screen on a phosphorimager and print the image as its actual size to use as a template for excision (Fig. 4a) (see Notes 1 and 2).

3.7. Excision of Bound RNAs

1. Place an image of the array under the slide and use it as a template to identify captured RNA. Add 0.4 µl of nanopure water to each position to be excised. Incubate for 30 s at room temperature, and then remove the unabsorbed water using a pipette. Using a toothpick, trace the circumference of the hydrated spot and remove it; once loosened, the gel slice will stick to the toothpick. Place the gel slice in a PCR tube.
2. Reexpose the slide to a phosphorimager screen overnight to ensure that the agarose containing bound RNAs was removed cleanly (without affecting neighboring spots) (Fig. 4a).

3.8. RT-PCR of Selected RNAs

1. Add 16 µl water, 2 µl 10× DNase buffer, and four units of DNase to each tube. Incubate the solution at 37°C for 2 h. Quench the reaction by the addition of 2 µl of 10× DNase stop solution (provided by Promega). Incubate at 65°C for 10 min to completely inactivate the DNase.

   Note: for a positive control, use approximately 100 fmoles of the original RNA library and set up the reaction under the same conditions.
2. Add 2 µl of 100 µM reverse primer (5′ CCT TGC GGA TCC AAT) to the tube. Anneal the RNA and DNA primer at 70°C for 10 min, and then incubate on ice for 10 min.
3. To complete reverse transcription, add 1.6 µl of 25 mM dNTPs, 0.8 µl of 10 mg/ml BSA, 4 µl of 10× RT buffer (provided by RT supplier), and two units of RT to +RT samples and the same volume of water for no RT controls. Incubate the reaction for 1 h at 60°C, and then inactivate the RT by heating at 95°C for 3 min.
4. To the above samples add 4 µl of 100 µM of forward primer (5′ GGC CGA ATT CTA ATA CGA CTC ACT ATA GGG AGA GGG TTT AAT), 2 µl of 100 µM of reverse primer, 0.6 µl of 250 mM $MgCl_2$, one unit of Taq DNA polymerase, 13 µl of H_2O, and 6 µl of 10× PCR buffer. Run 20–25 PCR cycles.
5. Set up a gel to determine if the RT-PCR was successful. Depending on the size of the PCR product, either an agarose or an acrylamide gel can be used to determine if the RT-PCR was successful. For acrylamide gels, a much smaller gel can be used: for example, 8.6 cm × 6.8 cm × 1 mm gel.
6. Mix 1 µl of RT-PCR reactions with 1 µl of 2× Loading Buffer. Load onto gel and run at 200–250 V for 30–45 min for acrylamide gels or at 100 V for 15–30 min for agarose gels.
7. Mix about 100 ml of 1× TBE buffer with 5 µl of 10 mg/ml aqueous ethidium bromide solution. (Alternatively, ethidium bromide can be added to agarose gels prior to pouring.) Soak

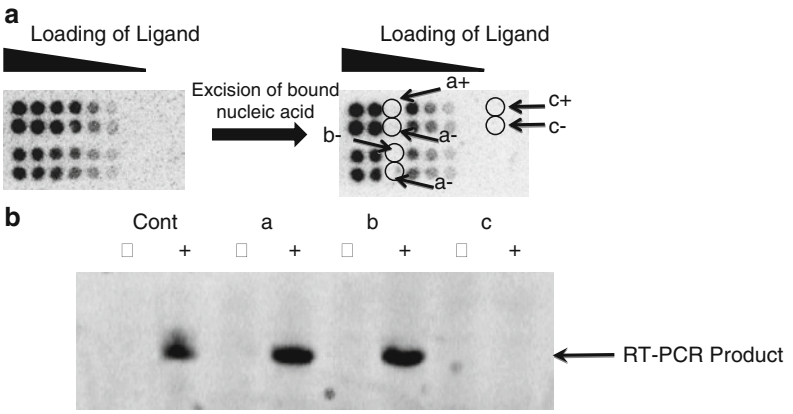


Fig. 4. Selection of RNA-ligand interactions on an array. A *left*, image of an array after hybridization with an RNA motif library. (**a**, *right*) Image of the array after the six indicated positions are excised. (**b**) Gel analysis of RT-PCR reactions completed with the samples that were excised. The *plus* indicates RT-PCR reactions in the presence of RT while the *minus* indicates RT-PCR reactions in the absence of RT.

the gel in this solution for 10 min and then image (Fig. 4b). Only experiments in which the +RT background samples (Fig. 4, position c) contain no product should be carried on toward cloning (see Notes 3 and 4).

3.9. Cell Culture and Cloning (32)

1. Steps 1–3 should be completed the day before transformation of cells (steps 4–6). Prepare LB broth as described above but add 15 g of agar before autoclaving. Autoclave on the liquid cycle at 121°C for 20 min. Remove the flask from autoclave, and cool the solution to about 60°C ensuring that the agar does not settle to the bottom (by swirling every 20 min). Add 0.05 g of Ampicillin dissolved in 1 ml of water; mix by swirling the flask.
2. Pour a thin layer (5 mm) of LB agar into a petri dish. Let each plate cool until the agar is solidified (about 20 min). Incubate the plates upside down at 37°C overnight. Store plates in plastic bags at 4°C.
3. Ligate PCR products into pGEM T Vector following the manufacturer's protocol (Promega).
4. DH5-α cells stored at −80°C should be thawed on ice for 5 min prior to use. Mix 100 µl DH5-α cells with 3 µl of ligation reaction in a 15 ml conical tube and incubate on ice for 30 min. Heat shock the cells at 42°C for 45 s, and then immediately place the tube on ice for 2 min. Add 1 ml of SOC media and incubate at 37°C with shaking (220 rpm) for 1 h.
5. While the transformed cells are shaking prepare the LB-agar plates for blue/white screening. Add 40 µl of 100 mM IPTG onto agar plate and spread evenly using a spreader bar. Close the lid and wait for 2 min for the solution to absorb into the

plates. Add 40 µl of 20 mg/ml X-gal; spread using the spreader. Place the plates in the 37°C incubator.

6. Plate 100 µl of transformed cells onto the plates prepared in step 2. Incubate plates at 37°C overnight (see Notes 5 and 6).
7. Transfer white colonies into separate wells of a deep 96-well plate containing 1 ml Terrific broth supplemented with 50 mg/l Ampicillin. (If it is difficult to determine if a colony is blue or white, place plates at 4°C for 30–60 min) Cover the 96-deep well plate with a piece of foil and grow the cells at 37°C with shaking (220 rpm) until they reach an $OD_{600} > 4$ (24–48 h). Wrap the LB-agar plates with parafilm and store at 4°C.
8. Prepare glycerol stocks of the cells cultured in step 7. It is easiest to use a multichannel pipette and a 96-well plate to prepare glycerol stocks. Add 100 µl of 50% glycerol (sterilized) to each well of a 96-well plate. Aseptically transfer 100 µl of the cells to the plate with glycerol. Mix by pipetting up and down; cover the plate and store at –80°C.
9. Centrifuge the 96-well deep well plate with the remaining culture to pellet the cells. Discard the supernatant by tipping the plate upside down and tapping gently on the bench top. Freeze cells at –80°C, and then send to a sequencing company.

4. Notes

1. If spots are faint or missing after hybridization of radiolabeled RNA, try one of the following:
   (a) Arrays might not be properly functionalized. Make new slides and try again.
   (b) Spotting solutions might be old. Make new spotting solution.
   (c) The hybridization buffer might be wrong. Check that the hybridization buffer was made properly.
   (d) Too few counts of the radiolabeled library might have been used. Radiolabel the RNA with a new stock of α-^{32}P ATP.
2. If the slides have high background, try one of the following:
   (a) Reactive groups were not quenched as $NaBH_3CN$ is a hygroscopic substance. If there are clumps in the solid $NaBH_3CN$, obtain a new stock.

(b) If slides were not prehybridized with BSA, repeat the experiment and prehybridize the slides with hybridization buffer containing 0.1% BSA.

3. If no bands observed on a gel after 25 cycles of RT-PCR for lanes with selected RNA, but bands are observed for positive control RT-PCR, it is possible that:
   (a) Bound RNA positions were not properly excised from the array. In this case, reimage the slide after excision to ensure that the proper spots were excised.
   (b) Too little RNA was harvested from the array. Excise a spot that captured more RNA (higher concentration of ligand loaded)
4. If the background spot or samples containing no reverse transcriptase amplify, the following might be a problem:
   (a) RT-PCR buffers are contaminated with DNA or RNA. To resolve this problem, obtain new stock solutions for RT-PCR.
5. If no colonies or very few colonies grow on an agar plate, try one of the following:
   (a) Poor transformation efficiency might be the cause. Ethanol precipitates the RT-PCR product and then ligates into vector.
   (b) Vector ligation was inefficient. Use more RT-PCR product in the vector ligation or use a new stock of DNA ligase to ligate into the vector.
6. If only blue colonies grow on agar plate, one of the following can cause the problem:
   (a) Vector ligation was inefficient. Try using more RT-PCR product in the vector ligation.
   (b) pGEM T or TOPO TA Vector is old. Obtain a fresh stock of vector and try again.
   (c) T4 DNA ligase is no longer active. Obtain a fresh stock of ligase and repeat the experiment.

Acknowledgments

We thank Professor Jessica Disney for careful proofreading of the manuscript. This work was supported by funding from the University at Buffalo, the NYS Center of Excellence and Bioinformatics and Life Sciences, a New Investigator Award from the Camille and Henry Dreyfus Foundation, a Cottrell Scholar Award from the Research Corporation, a NYSTAR J. D. Watson Young Investigator Award, and the National Institutes of Health (GM079235).

References

1. Ellington, A. D., and Szostak, J. W. (1990) In vitro selection of RNA molecules that bind specific ligands, *Nature* **346**, 818–822.
2. Ellington, A. D., and Szostak, J. W. (1992) Selection in vitro of single-stranded DNA molecules that fold into specific ligand-binding structures, *Nature* **355**, 850–852.
3. Osborne, S. E., and Ellington, A. D. (1997) Nucleic acid selection and the challenge of combinatorial chemistry, *Chem. Rev.* **97**, 349–370.
4. Tuerk, C., and Gold, L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, *Science* **249**, 505–510.
5. Cox, J. C., and Ellington, A. D. (2001) Automated selection of anti-protein aptamers, *Bioorg. Med. Chem.* **9**, 2525–2531.
6. Giver, L., Bartel, D. P., Zapp, M. L., Green, M. R., and Ellington, A. D. (1993) Selection and design of high-affinity RNA ligands for HIV-1 REV, *Gene* **137**, 19–24.
7. Hesselberth, J. R., Miller, D., Robertus, J., and Ellington, A. D. (2000) In vitro selection of RNA molecules that inhibit the activity of ricin A-chain, *J. Biol. Chem.* **275**, 4937–4942.
8. Daniels, D. A., Chen, H., Hicke, B. J., Swiderek, K. M., and Gold, L. (2003) A tenascin-C aptamer identified by tumor cell SELEX: Systematic evolution of ligands by exponential enrichment, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 15416–15421.
9. Minunni, M., Scarano, S., and Mascini, M. (2008) Affinity-based biosensors as promising tools for gene doping detection, *Trends Biotechnol.* **26**, 236–243.
10. Famulok, M., Hartig, J. S., and Mayer, G. (2007) Functional aptamers and aptazymes in biotechnology, diagnostics, and therapy, *Chem. Rev.* **107**, 3715–3743.
11. Wu, C. C., Sabet, M., Hayashi, T., Tawatao, R., Fierer, J., Carson, D. A., Guiney, D. G., and Corr, M. (2008) In vivo efficacy of a phosphodiester TLR-9 aptamer and its beneficial effect in a pulmonary anthrax infection model, *Cell Immunol.* **251**, 78–85.
12. Keefe, A. D., and Schaub, R. G. (2008) Aptamers as candidate therapeutics for cardiovascular indications, *Curr. Opin. Pharmacol.* **8**, 147–152.
13. Lorsch, J. R., and Szostak, J. W. (1994) In vitro evolution of new ribozymes with polynucleotide kinase activity, *Nature* **371**, 31–36.
14. Bartel, D. P., and Szostak, J. W. (1993) Isolation of new ribozymes from a large pool of random sequences [see comment], *Science* **261**, 1411–1418.
15. Mendonsa, S. D., and Bowser, M. T. (2004) In vitro evolution of functional DNA using capillary electrophoresis, *J. Am. Chem. Soc.* **126**, 20–21.
16. Klug, S. J., and Famulok, M. (1994) All you wanted to know about SELEX, *Mol. Biol. Rep.* **20**, 97–107.
17. Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray, *Science* **270**, 467–470.
18. MacBeath, G., and Schreiber, S. L. (2000) Printing proteins as microarrays for high-throughput function determination, *Science* **289**, 1760–1763.
19. Houseman, B. T., and Mrksich, M. (2002) Carbohydrate arrays for the evaluation of protein binding and enzymatic modification, *Chem. Biol.* **9**, 400–401.
20. Barrett, O. J., Childs, J. L., and Disney, M. D. (2006) Chemical microarrays to identify ligands that bind pathogenic cells, *Chembiochem* **7**, 1882–1885.
21. Disney, M. D., Magnet, S., Blanchard, J. S., and Seeberger, P. H. (2004) Aminoglycoside microarrays to study antibiotic resistance, *Angew. Chem. Int. Ed.* **43**, 1591–1594.
22. Bradner, J. E., McPherson, O. M., and Koehler, A. N. (2006) A method for the covalent capture and screening of diverse small molecules in a microarray format, *Nat. Prot.* **1**, 2344–2352.
23. Duffner, J. L., Clemons, P. A., and Koehler, A. N. (2007) A pipeline for ligand discovery using small-molecule microarrays, *Curr. Opin. Chem. Biol.* **11**, 74–82.
24. MacBeath, G., Koehler, A. N., and Schreiber, S. L. (1999) Printing small molecules as microarrays and detecting protein-ligand interactions en masse, *J. Am. Chem. Soc.* **121**, 7967–7968.
25. Afanassiev, V., Hannemann, V., and Wolfl, S. (2000) Preparation of DNA and protein micro arrays on glass slides coated with an agarose film, *Nucleic Acids Res.* **28**, E66.
26. Dufva, M., Petronis, S., Jensen, L. B., Krag, C., and Christensen, C. B. (2004) Characterization of an inexpensive, nontoxic, and highly sensitive microarray substrate. *Biotechniques* **37**, 286–292, 294, 296.

27. Childs-Disney, J. L., Wu, M., Pushechnikov, A., Aminova, O., and Disney, M. D. (2007) A small molecule microarray platform to select RNA internal loop-ligand interactions, *ACS Chem. Biol.* **2**, 745–754.
28. Disney, M. D., Labuda, L. P., Paul, D. J., Poplawski, S. G., Pushechnikov, A., Tran, T., Velagapudi, S. P., Wu, M., and Childs-Disney, J. L. (2008) Two-dimensional combinatorial screening identifies specific aminoglycoside-RNA internal loop partners, *J. Am. Chem. Soc.* **130**, 11185–11194.
29. Chan, T. R., Hilgraf, R., Sharpless, B., and Fokin, V. V. (2004) Polytriazoles as copper(I)-stabilizing ligand in catalysis, *Org. Lett.* **6**, 2853–2855.
30. Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001) Click chemistry: diverse chemical function from a few good reactions, *Angew. Chem. Int. Ed.* **40**, 2004–2021.
31. Milligan, J. F., and Uhlenbeck, O. C. (1989) Synthesis of small RNAs using T7 RNA polymerase, *Methods Enzymol.* **180**, 51–62.
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning*, Vol. 1, Cold Spring Harbor, NY.
33. Peyret, N., Seneviratne, P. A., Allawi, H. T., and SantaLucia Jr, J. (1999) Nearest-neighbor thermodynamics and NMR of DNA sequences with internal A-A, C-C, G-G, and T-T mismatches, *Biochemistry* **38**, 3468–3477.
34. SantaLucia Jr, J. (1998) A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1460–1465.

INDEX

Mahesh Uttamchandani and Shao Q. Yao (eds.), *Small Molecule Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 669,
DOI 10.1007/978-1-60761-845-4, © Springer Science+Business Media, LLC 2010

F

G

H

I

K

L

M

N

O

P

Q

R

S

T

U

V

W