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Microarrays for the Functional Analysis of the Chemical-Kinase Interactome

KURUMI Y. HORIUCHI,[1] YUAN WANG,[1] SCOTT L. DIAMOND,[2] and HAICHING MA[1]

A central challenge in chemical biology is profiling the activity of a large number of chemical structures against hundreds of biological targets, such as kinases. Conventional ^{32}P-incorporation or immunoassay of phosphorylated residues produces high-quality signals for monitoring kinase reactions but is difficult to use in high-throughput screening (HTS) because of cost and the need for well-plate washing. The authors report a method for densely archiving compounds in nanodroplets on peptide or protein substrate-coated microarrays for subsequent profiling by aerosol deposition of kinases. Each microarray contains over 6000 reaction centers (1.0 nL each) whose phosphorylation progress can be detected by immunofluorescence. For $p60^{c\text{-}src}$, the microarray produced a signal-to-background ratio of 36.3 and Z′ factor of 0.63 for HTS and accurate enzyme kinetic parameters (K_m^{ATP} = 3.3 µM) and IC_{50} values for staurosporine (210 nM) and PP2 (326 nM) at 10 µM adenosine triphosphate (ATP). Similarly, B-Raf phosphorylation of MEK-coated microarrays was inhibited in the nanoliter reactions by GW5074 at the expected IC_{50} of 9 nM. Common kinase inhibitors were printed on microarrays, and their inhibitory activities were systematically profiled against B-Raf (V599E), KDR, Met, Flt-3 (D835Y), Lyn, EGFR, PDGFRβ, and Tie2. All results indicate that this platform is well suited for kinetic analysis, HTS, large-scale IC_{50} determinations, and selectivity profiling. (*Journal of Biomolecular Screening* 2006:48-56)

Key words: chemical microarray, kinase profiling, functional assay, high-throughput screening (HTS), ELISA

INTRODUCTION

Protein kinases are a large superfamily of homologous proteins with 518 members in the human genome. The majority phosphorylate serine and threonine residues, whereas ~90 kinases phosphorylate tyrosine residues.[1] Phosphorylation events help to regulate basic cell signaling networks, and thus kinase dysfunction is a key factor in diseases such as cancer, inflammation, and diabetes.[2] The introduction of Gleevec (an adenosine triphosphate [ATP]–competitive inhibitor of Bcr-Abl for chronic myelogenous leukemia) demonstrated that kinases are drugable targets. About 24% of research and development (R&D) spending in pharmaceutical industries focuses on kinases.[3] Because only ~7% of kinases have available crystal structures and their substrate and inhibitor binding motifs are similar, rational drug design for kinase has proven difficult. High-throughput screening (HTS) of diversified chemical libraries provides a major means for identification of lead candidates for drug development.

Common analytical and HTS assay methods for kinases have used both homogeneous and heterogeneous platforms. Separation-free, homogeneous kinase assays include florescence polarization (FP), time-resolved fluorescence resonance energy transfer (TR-FRET), enzyme fragmentation complementation assay (EFC), and luciferase detection of residual ATP. These assays are easily adapted for well-plate HTS, but optically active compounds and fluorescence labeling of substrates can generate various mechanisms of interference. As a result, when several different assays are used against the same library and target, there is a striking lack of correspondence in the set of inhibitors (hits) found.[4] Heterogeneous platforms include ^{32}P-ATP or enzyme-linked immunosorbent assay (ELISA)–based detection, and these assays are the gold standard for kinase profiling and offer excellent sensitivity with no fluorescence interference. However, the requirement of separation and/or multiple washing steps limits their use in high-throughput applications. Total costs for radioisotope usage are also substantial. These drawbacks of heterogeneous assays are potentially minimized or eliminated when HTS is conducted on a microarray format that 1) reduces reagent consumption (compound, kinase, substrate, and detector reagents), 2) eliminates compound interference, and 3) allows automation of multiple wash steps.

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The development of chemical microarrays is fairly recent.[5-7] One of the key problems is that compounds are ideally screened in solution phase. When reactions are reduced to a microarray format, no automatic liquid-handling machine can reliably activate these reactions individually. As a result, compounds are either immobilized on a microarray surface by direct linkage[5,6] or maintained as dried spots for subsequent rehydration by gel overlay.[7] Only a limited number of chemical scaffolds have been linked on microarrays for binding-based screening.[8] Given the large size and number of existing chemical libraries, no universal linkage is feasible. Houseman et al[9] advanced the use of microarrays for kinase screening by linking peptide substrates on the surface of microarray and then spotting a mixture, including chemical compound, kinase, and ATP, on top of the coating. They demonstrated that both phosphorylation and inhibition reactions can be detected in this heterogeneous format. However, this 2-step process of premixing reaction components in well-plates and postreaction on microarray does not reduce the costs of the kinase or facilitate the profiling of tens of thousands of compounds against scores of kinases because liquid-handling burdens are the same as classic HTS.

We have developed a new microarray method for high-throughput analysis of the chemical-kinase interactome based on the use of aerosol deposition of reagents into glycerol-enriched nanodroplets containing chemical compounds.[10,11] Here we introduce a kinase screening method that always maintains the kinase and compound in nanoliter liquid droplets and allows for easy subsequent detection of an immobilized phosphorylated substrate via immunofluorescence staining. Evaporation of the reaction, before and after initiation with the kinase, is prevented by adding 10% glycerol into a typical buffer as the arraying solution.[10,11] Humidity incubation always keeps reactions to proceed at a final concentration of 90% water by volume. This format reduces consumables by more than 90% and facilitates the creation of numerous sets of a compound library for kinase HTS or profiling. This is an advantage when each compound is only available in small quantities and profiling data are needed prior to a decision to conduct resynthesis.

MATERIALS AND METHODS

Reagents

Kinases used in this study were purchased from Upstate (Lake Placid, NY): Src (p60$^{c\text{-}Src}$, Upstate cat. 14-117), expressed in Sf9 insect cells by recombinant baculovirus containing the human *c-src* gene; Lyn (Upstate cat. 14-510), N-terminal His_6-tagged recombinant full-length human Lyn expressed in Sf21 cells; B-Raf (V599E, Upstate cat. 14-557), N-terminal GST-tagged recombinant human B-Raf residues 416-end expressed in Sf21 cells; Flt3 (D835Y, Upstate cat. 14-610), N-terminal GST-tagged recombinant human Flt3 residues 564-end expressed in Sf21 cells; PDGFRβ (Upstate cat. 14-463), N-terminal His_6-tagged recombinant human PDGFRβ residues 557-end expressed in Sf21 cells; EGFR (Upstate cat. 14-531), N-terminal GST-tagged recombinant human EGFR residues 696-end expressed in Sf21 cells; c-Met (Upstate cat. 14-526), N-terminal His_6-tagged recombinant human Met residues 974-end expressed in Sf21 cells; KDR (Upstate cat. 14-630), N-terminal His_6-tagged recombinant human KDR residues 790-end expressed in Sf21 cells; and Tie2 (Upstate cat. 14-540), N-terminal His_6-tagged recombinant human Tie2 residues 771-end expressed in Sf21 cells. Peptide substrates, inactive MEK1, biotinylated poly (Glu4-Tyr)$_{10}$, abltide, and anti-phospho-MEK were also purchased from Upstate (Lake Placid, NY). Phosphotyrosine standard peptide, Biotin-RRLIEDAEpYAARG-NH_2, was from AnaSpec, Inc. (San Jose, CA). Protein slides to immobilize proteins were purchased from Full Moon Biosystems (Sunnyvale, CA). Epoxy slides were obtained from TeleChem (Sunnyvale, CA). Streptavidin-coated slides were from Xenopore Corp. (Hawthorne, NJ). ATP (Adenosine 5′-triphosphate disodium salt, cat. A7699), bovine serum albumin (BSA, cat. A7906), staurosporine (cat. S5921), and other general chemicals were purchased from Sigma. PP2 (AG 1879, 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine), GW5074, KDR inhibitor ((Z)-3-[(2,4-Dimethyl-3-(ethoxycarbonyl)pyrrol-5-yl)methylidenyl]indolin-2-one), and PDGFR inhibitor 1 (D-64406) (5-Hydroxy-1H-2-indolyl)(1H-2-indolyl)-methanone) were from Calbiochem (San Diego, CA). Primary antibodies for phosphotyrosine, P-Tyr-100, and biotinylated substrate for cMet, Pyk2 with core sequence of -DIYAE-, were purchased from Cell Signaling (Beverly, MA); secondary antibodies, Alexa Fluor 555-labeled goat anti-rabbit and anti-mouse IgG, were from Molecular Probes (Eugene, OR).

Coating of microarray surface

For protein coating of microarrays, protein-active slides were precoated by single 50-µL/slide dispense of 1 µM MEK in phosphate-buffered saline (PBS) to the center of the slide, which was then spread out by covering with a hybridization cover slip (LifterSlip, Erie Scientific Company, Portsmouth, NH) and then incubated at room temperature for 2 h in a humidified chamber with > 90% R_h (relative humidity). Slides were then washed with PBS, rinsed with double-distilled water, and spun dry prior to microarraying. For coating MEK, the native substrate of Raf kinase, Full Moon Biosystems Protein slides were found to bind up to 500 nM of MEK in the printing solution (compared to 300 nM on the epoxy slide). Streptavidin-coated slides were coated with biotinylated peptides in a similar manner as above; the substrates were poly-(Glu$_4$-Tyr)$_{10}$ for KDR, Src, Lyn, EGFR, PDGFRβ, and Tie2; biotinylated abltide for Flt3; and biotinylated Pyk2 for Met.

Chemical library arraying and kinase assay

Chemical compounds in pure DMSO were diluted 1:50 into kinase reaction buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij 35, 10% glycerol, 2 mM

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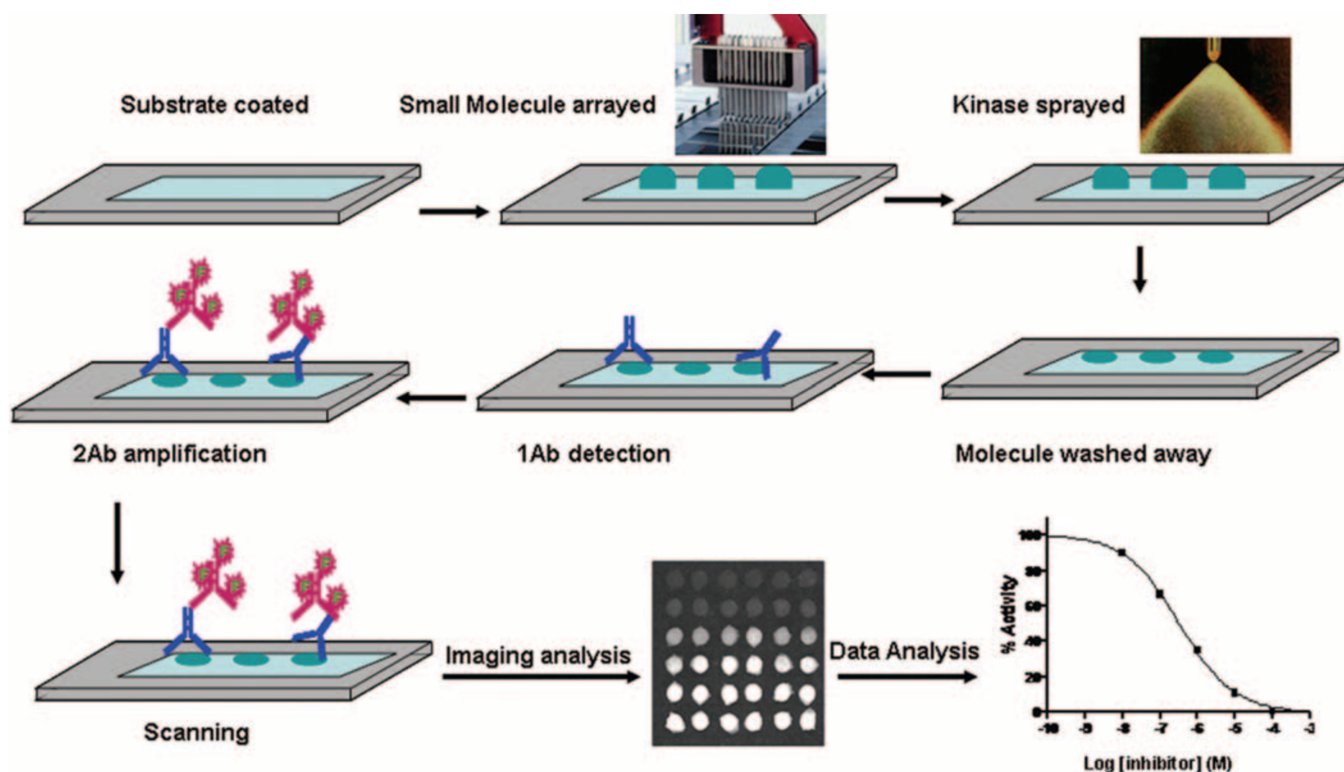

FIG. 1. Determination of chemical-kinase interactions on a microarray. Protein- or peptide-based kinase substrates were first immobilized by being coated on the surface of the microarray, small-molecule chemicals were arrayed, and the microarrays were activated by spray deposition of the kinase. Slides were washed, and phosphorylated immobilized substrates were detected by immunofluorescence. Spot intensities allow determination of the extent of reaction for a given 1-nL kinase reaction.

DTT, 0.1 mM Na_3VO_4, and 0.02 mg/mL BSA with different concentrations of ATP. The final printing solution contained 2% DMSO and 10% glycerol in a 384-well plate and then arrayed on substrate-coated slides. Arrayed features were 120 to 150 µm in diameter with a 350-µm center-to-center distance. Kinases were deposited as a fine aerosol with an average droplet size of ~18 µm in diameter and 2.2 pL in volume as previously described.[11] After printing the reaction mixture on slides, kinases such as $p60^{c\text{-}Src}$ and B-Raf (V599E) were diluted into a solution containing 0.1 mg/mL BSA and deposited on slides by the sprayer. The glycerol-enriched features were then fully hydrated to a final glycerol concentration of 10% by volume in a controlled incubator at 30 °C/96% R_h. Slides were then washed in stopping buffer containing 50 mM EDTA/TPBS (PBS and 0.05% Tween-20) to stop the reactions and blocked in a sealed slide holder, which was continuously rotated with Labquake® (Barnstead International, Dubuque, IA) with a blocking buffer that contained 10 mg/mL BSA in TPBS for 1 h at room temperature (automatic slide washer can also be used for a large quantity of slide processing). After washing in PBS, slides were treated with the primary antibody, mouse monoclonal P-Tyr-100, for phosphotyrosine detection or rabbit anti-phospho-MEK1 (Ser218/22)/MEK2 (Ser 222/226) antibody for B-Raf (V599E) reaction, diluted to 1:400 in an antibody dilution buffer containing 5 mg/mL BSA in TPBS for 1 h at room temperature. After washing 3 times for 10 min, slides were then treated with Alexa Fluor 555–labeled secondary antibody for 1 h at room temperature in the dark. Slides were then washed in the antibody dilution buffer (3 times for 10 min), 1 time with distilled water, and spun dry. Microarrays were scanned in a GenePix 4100A (Axon Instruments) at Ex = 532 m, with a 575±25 nm filter for emission. The image of the microarray was converted to signal by using DNA microarray software of Acuity (Axon Instruments) or ArrayPro (Media-Cybernetics, Silver Spring, MD). Nonlinear regression and plotting to obtain IC_{50} and K_m were performed with Graphpad Prism (San Diego, CA).

RESULTS

Microarray analysis of kinase reactions

Microarrays were coated with the kinase substrate followed by microarraying of chemical compounds in 10% glycerol kinase reaction buffer and subsequent activation with kinase (**Fig. 1**). ATP can be added with the chemical compound or delivered with the kinase. Alternatively, inexpensive biotinylated peptides can be added to the compound well prior to printing on streptavidin-

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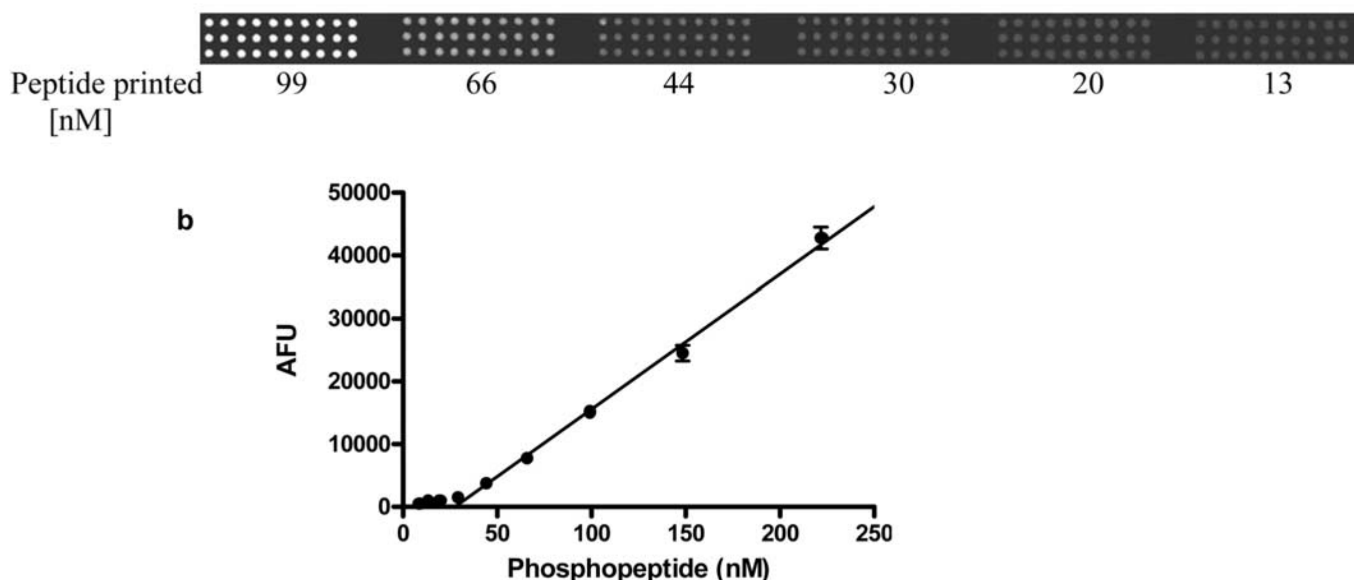

FIG. 2. Phosphopeptide standard curve using immunofluorescence detection. Biotinylated phosphopeptide (Biotin-RRLIEDAEpYAARG-NH_2) was immobilized on streptavidin-coated slides by printing at different concentrations in kinase reaction buffer. Slides were then blocked and immunostained and imaged with a laser scanner (**a**) to yield a calibration curve with wide linear dynamic range (**b**).

coated slides. After incubating the microarrays under controlled temperature and humidity, the microarrays can be washed with antibody dilution buffer, immunostained, and laser scanned. The spot intensity is then quantified. We have successfully applied this approach to more than 20 serine/threonine and tyrosine kinases, including receptor kinases, with the reaction times from 15 min to overnight incubation and ATP concentrations ranging from 2 to 100 µM.

To determine the detection sensitivity of immunofluorescence detection and laser scanning, biotinylated phosphopeptide (Biotin-RRLIEDAEpYAARG-NH_2) was microarrayed on streptavidin-coated slides by printing at different concentrations (**Fig. 2a**). The detection limit of ~10 nM in the spot was obtained (**Fig. 2b**) with a linear dynamic range from 20 to 200 nM of printed phosphopeptide calibrant. The detection limit can be lowered by ~10-fold with tyramide signal amplification (not shown).

HTS of $p60^{c\text{-}src}$ and B-Raf kinase

To evaluate the signal quality and uniformity of kinase reactions in HTS mode, we have performed the Src ($p60^{c\text{-}src}$) reactions on a microarray with biotinylated poly-(Glu_4-Tyr)$_{10}$ peptide (**Fig. 3**). A total of 3000 reactions of 10% glycerol kinase reaction buffer, including biotinylated substrate and 50 µM ATP with or without 100 µM staurosporine, were arrayed on a streptavidin-coated slide. We have used high concentrations of ATP and staurosporine in the assays to drive both phosphorylation and inhibition into completions. The slide was activated by spray deposition of 0.25 U/µL of $p60^{c\text{-}src}$ kinase (10 U to activate 3000 reactions). The phosphorylated substrate in each reaction center was then detected by immunofluorescence staining to obtain a signal-to-background ratio (0 µM inhibitor/100 µM inhibitor) of 36.3 and $Z' = 0.63$.

The reactions of $p60^{c\text{-}src}$ kinase produced a dose- and time-dependent signal (**Fig. 4a**), thus allowing enzyme kinetic analysis (Fig. 4b) and dose-response inhibition assays (**Fig. 4c,d**). For kinetic studies of $p60^{c\text{-}src}$ using poly-(Glu_4-Tyr)$_{10}$ as a substrate, reaction buffers with 6 different ATP concentrations were arrayed on replicate substrate-coated microarrays and activated with the $p60^{c\text{-}src}$. All microarrays were then incubated (30 °C, 96% R_h), stopped at different time points by washing slides in PBS containing 50 mM EDTA, and followed by immunofluorescence detection (**Fig. 4a**). The fluorescence signal at each time point was converted into the level of phosphorylation by using the phosphopeptide standard shown in **Figure 2**. The ATP dose-dependent $p60^{c\text{-}src}$ reactions indicated that ATP was rate limiting for phosphorylation of substrate (**Fig. 4a,b**). The K_m value of ATP derived by initial rate analysis was 3.3 µM (**Fig. 4b**), which is in full agreement with earlier reports of 2.2 to 4 µM.[12,13] With a similar approach, we determined the IC_{50} values of staurosporine and PP2. Staurosporine is an ATP-competitive inhibitor that potently inhibits a broad range of kinases, including Src,[14,15] and PP2 is an ATP-noncompetitive inhibitor that is specific for Src family kinases.[16-18] Our studies showed that the IC_{50} values of staurosporine were 210 nM and 2100 nM at ATP concentrations of 10 µM and 100 µM, respectively, demonstrating ATP-competitive inhibition (**Fig. 4c**).

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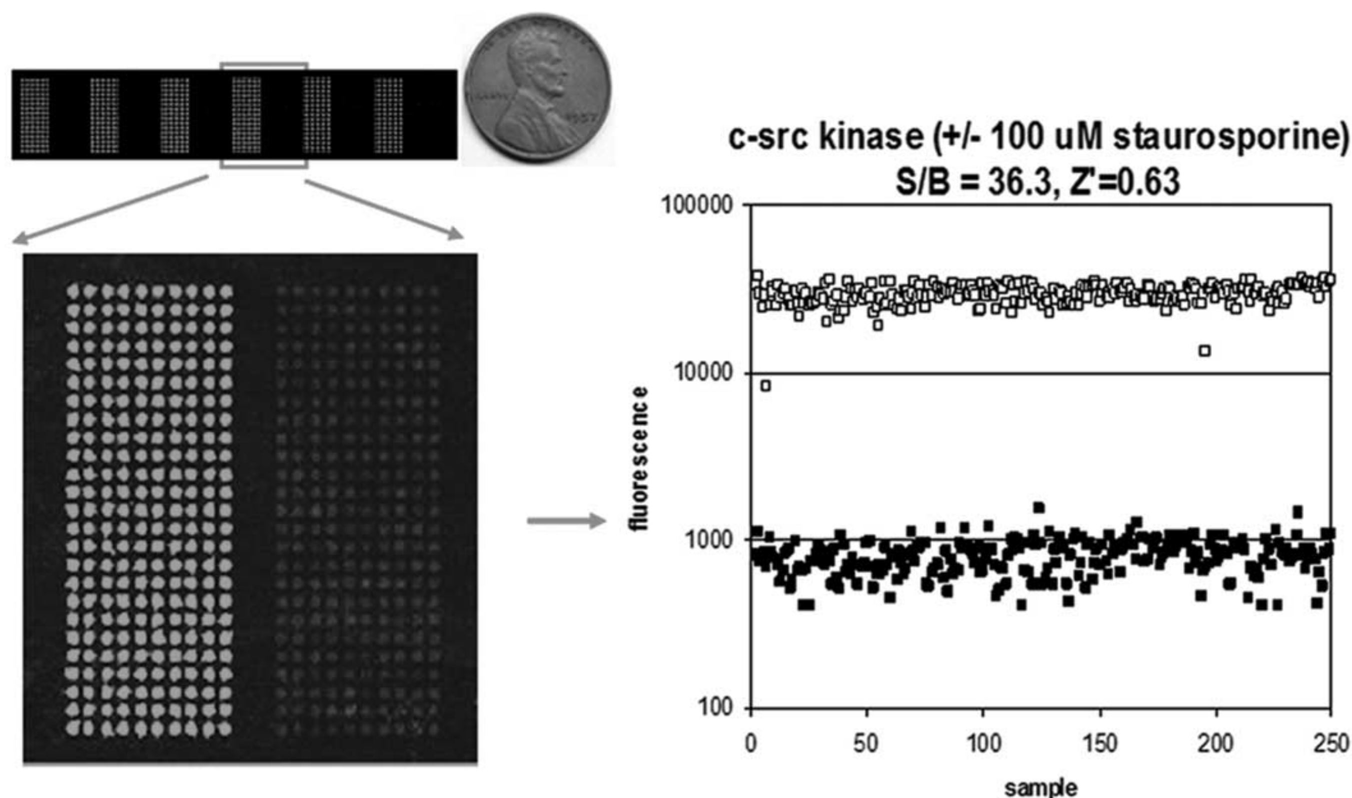

FIG. 3. High-throughput screening of the p60[c-src] assay at 50 µM adenosine triphosphate (ATP) in the absence or presence of 100 µM staurosporine. Src reaction was performed by printing substrate (biotinylated poly(Glu_4-Tyr)$_{10}$) in the kinase reaction buffer, as well as 50 µM ATP in the presence or absence of 100 µM staurosporine, on streptavidin-coated slide. Reactions were activated by spraying Src kinase (p60[c-src]) onto the microarray (10 units/slide). After incubation at 30 °C with 96% humidity for 2 h, reactions were detected and imaged. Full signal without inhibitor was 29,209 (12% coefficient of variation [CV]), and the signal in the presence of the inhibitor was 805 (5.6% CV) (signal/background = 36.3).

On the other hand, the IC_{50} values of PP2 were around 450 nM at both ATP concentrations at 10 µM and 100 µM, indicating ATP-noncompetitive inhibition (**Fig. 4d**).

The Ras/Raf/MAPK signaling cascade is a classic pathway known for mitogenesis and mediating cellular proliferation in various cell types. B-Raf mutation is a common cause of melanoma, and the V599E mutant is constitutively active without the requirement of upstream G-protein Ras.[19] Unfortunately, no peptide-based substrate is suitable for assay development. Therefore, the native, inactive kinase MEK protein was chosen as a substrate and coated on a protein-reactive microarray. B-Raf (V599E) was then tested with the Raf kinase inhibitor, GW5074.[20] Under ATP concentrations ranging from 10 to 100 µM, the IC_{50} value of each ATP concentration was ~9 nM (**Fig. 5**), in agreement with the published value[20] and indicative of ATP-noncompetitive inhibition by GW5074.

Kinase selectivity profiling

Finding specific kinase inhibitors without cross-reactivity is a major challenge, especially for ATP-competitive inhibitors, because all kinases use ATP as a substrate.[1] One distinct advantage of microarrays is the capability to print multiple sets of the same library to perform compound selectivity profiling against a panel of kinases. Each microarray slide can be precoated with an individual substrate paired for intended use with a matched kinase deployed in the spray activation step. We have profiled a small proprietary compound library against multiple kinases (**Fig. 6a**), and selective activities were clearly demonstrated through the 4 control compounds—KDRI, GW5074, PDGFR I-1, and staurosporine, each arrayed in duplicate at 10 µM concentration on all microarrays (**Fig. 6b, Table 1**). Following HTS of the library (**Fig. 6a**) with a hit rate against B-raf set at 1% (≥ 70% inhibition cutoff), we then achieved a hit confirmation rate of 77% by IC_{50} determination on the microarrays. The enlarged images show the same position on the microarrays tested against 8 different kinases. Each compound had its preferred targets. For example, GW5074 is more specific for B-Raf (V599E), whereas PDGFRI is more potent for PDGFRβ, KDR and Flt3 (D835Y), and staurosporine strongly inhibited all the kinases tested (**Table 1**). This demonstrates that a large series of molecules (thousands per microarray or hundreds of IC_{50} dose-dilution series) can be printed on replicate microarrays, each uniquely precoated with an optimal substrate for kinase profiling.

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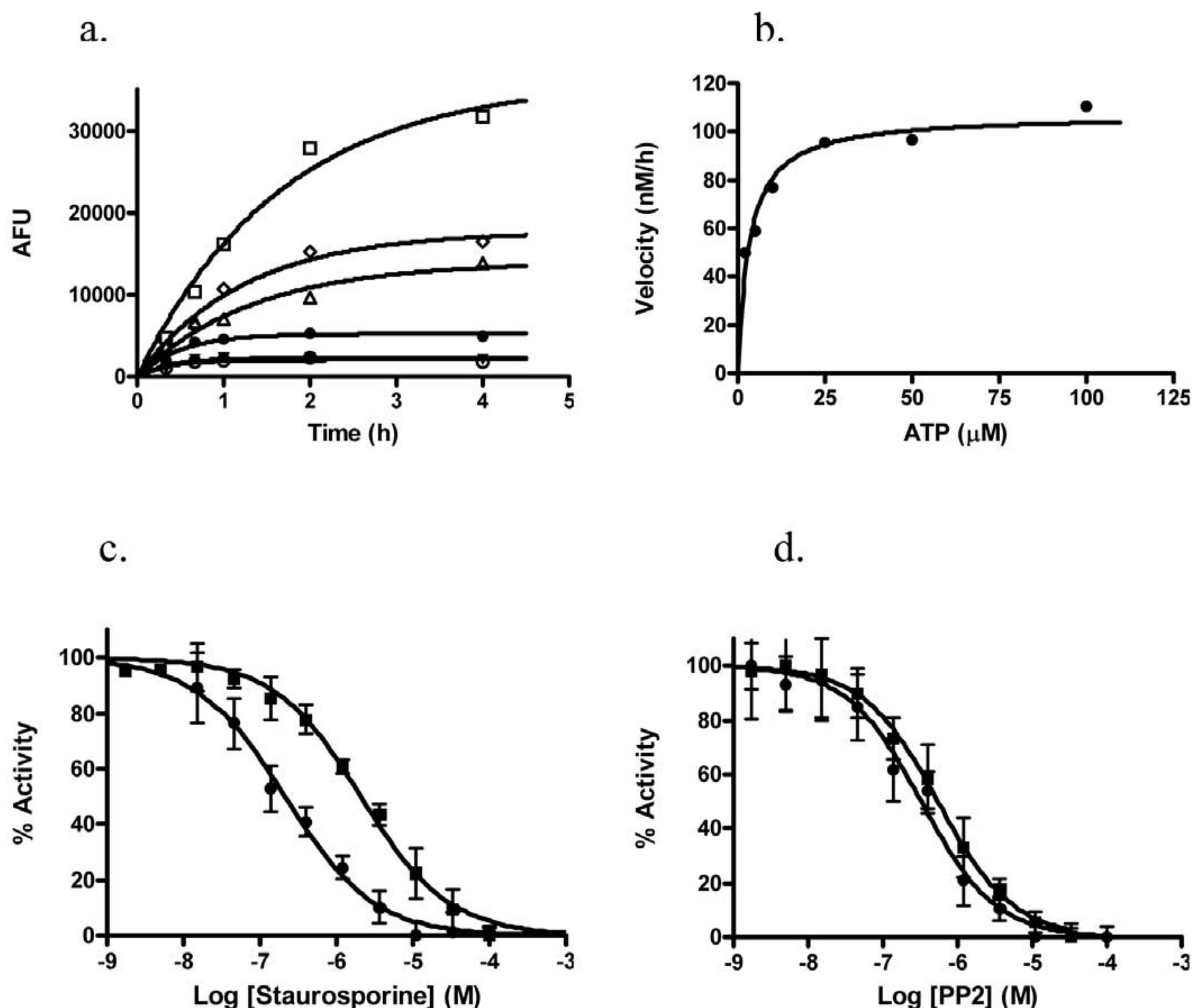

FIG. 4. Kinetic analysis of p60$^{c\text{-}src}$ reactions on a microarray. (**a**) Time-dependent c-Src reaction was performed similar to that described in **Figure 3**, with different concentrations of adenosine triphosphate (ATP) at 2 μM (open circles), 5 μM (open inverted triangles), 10 μM (solid circles), 25 μM (open triangles), 50 μM (open diamonds), and 100 μM (open squares). Initial velocities obtained from slopes of the linear phase of the time courses were plotted against ATP concentrations. (**b**) Kinetic constants obtained from nonlinear regression were 3.3 μM and 107 nM/h for ATP K_m and V_{max}, respectively. IC_{50} values of inhibitors staurosporine and PP2 at different concentrations of ATP were determined. The IC_{50} values are (**c**) 210 nM and 2.1 μM for staurosporine at 10 and 100 μM ATP, respectively, and (**d**) 326 and 540 nM for PP2 at 10 and 100 μM ATP, respectively.

DISCUSSION

Microarray technology has been successfully used as a basic research tool in genomics, protein function analysis,[21] and analyzing proteome activities in yeast.[22,23] New applications have slowly extended into the drug discovery process, such as the toxicity assay chip for predicting drug toxicity.[24] In this study, we have created a small-molecule microarray that can be directly used for HTS and follow-up IC_{50} hit verification, as well as mechanistic studies and compound selectivity profiling against kinases. The unique aerosol deposition technology allows minute quantities of kinase to activate thousands of reactions. In comparison to well-plate methods, the microarray has several advantages. First, the method allows the traditional solution phase biochemical reactions without physically linking or caging chemical compound or kinases on the microarray surface. Therefore, this technology can be broadly used for any existing chemical library. Compound optical interference is not present because the reaction step is decoupled from the

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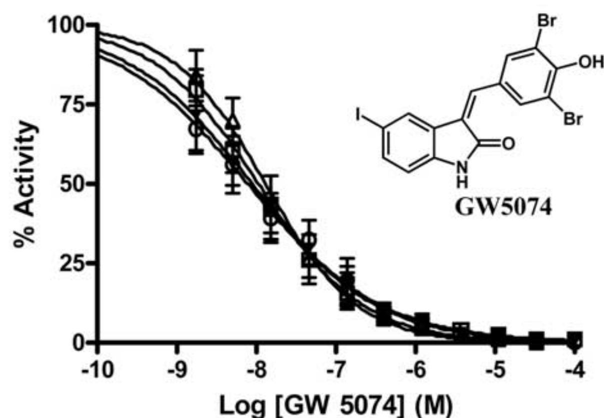

FIG. 5. Dose-response inhibition of B-Raf (V599E) kinase reaction on a microarray. The substrate of inactive MEK1 was precoated on protein slides, and inhibitor in serial dilutions was mixed with different adenosine triphosphate (ATP) concentrations and arrayed on a substrate-coated microarray. Subsequent reaction was performed as mentioned in the Methods section, with a B-Raf concentration of 10 units/slide. IC_{50} values were 12 nM, 6.9 nM, 7.7 nM, and 9.5 nM for GW5074 at 10, 25, 50, and 100 µM ATP, respectively.

detection step by microarray washing. Immunodetection provides for a high specificity signal that is amenable to enzyme amplification via precipitated fluorescent substrates. Also, laser scanners are generally more sensitive than plate readers. In terms of throughput, aerosol deposition can activate 10 microarrays in 20 min, with each array containing more than 6000 reactions. In contrast, microfluidic lab-on-a-chip platforms run ~10 to 30 reactions per chip.[25] Reagent savings are also significant with microarrays. For example, in a conventional 384-well–based format, average reagent cost for a typical kinase reaction is between $1 and $1.50 for the ELISA-based assay, and the cost for the homogeneous-based assay is slightly higher in our calculation. However, the average reagent cost can be reduced 10-fold with microarrays. Highly parallel array printing allows screening of replicate microarrays without an increase in the initial liquid handling to create the print plates (as in **Fig. 6**). This platform does not require sophisticated micropositioning or clog-prone piezo-dispensing, while maintaining compatibility with a variety of assay components. The common biotin-streptavidin interaction is used here in all the experiments for peptide immobilization. However, because all had an ELISA-based format, the length of a selected linker used to link

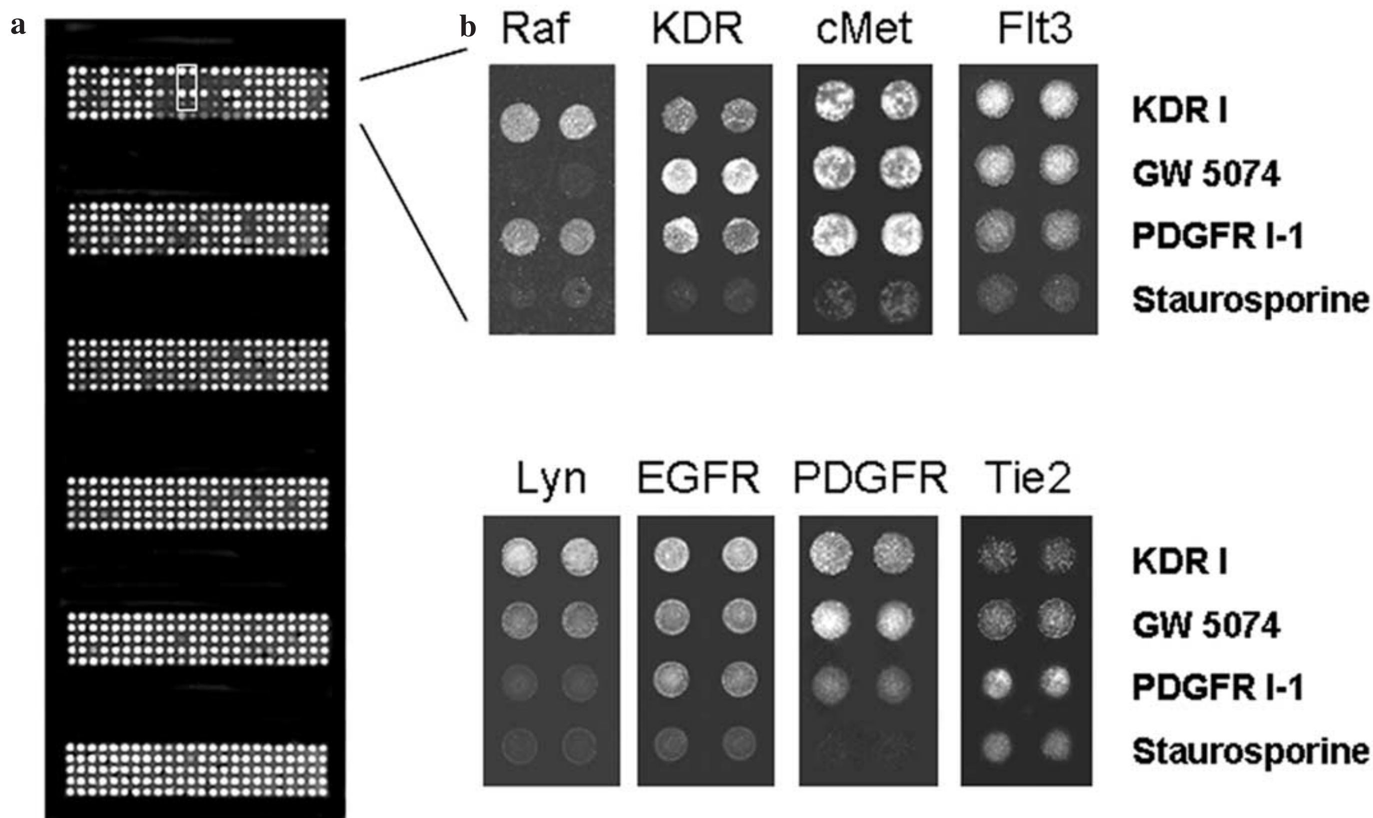

FIG. 6. (**a**) Specificity profiling against multiple kinase targets. The left-side microarray image shows a part of the 6000 B-Raf (V599E) reactions on the chip printed with a small compound library. (**b**) The enlarged images show the same portion of array from different kinase profile screenings, which contained duplicates of each of the 4 control inhibitors; the inhibition activities are listed in **Table 1**. Both compound and adenosine triphosphate (ATP) concentrations were 10 µM.

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Table 1. Specificity of Inhibitors for Kinase Profiling

	% Inhibition			
Kinases	*KDRI*	*GW5074*	*PDGFR I-1*	*Staurosporine*
B-Raf (V599E)	0	100	67	94
KDR	75	9	85	100
Met	6	6	0	76
Flt3 (D835Y)	70	77	89	100
Lyn	0	22	50	67
EGFR	20	24	17	74
PDGFRβ	61	52	87	98
Tie2	43	31	5	36

peptide to the reaction surface (chip or plate) may affect assay conditions.

The printing buffer used for fabricating the microarrays is the same as the conventional enzyme reaction except 10% glycerol was added to prevent complete spot evaporation. Glycerol is commonly used for enzyme storage and enzyme reactions. High concentrations of viscosogens, such as glycerol, trehalose, and sucrose, have been reported to decrease the V_{max} value for kinases[26,27] and H^+-ATPase,[28] but the effects are relatively small at low concentrations of viscosogens.[29] We have also tested the glycerol effect on many enzyme activities, such as Akt-1, EGFR, FGFR, Flt3 (D835Y), Fyn, IRK, KIT, LCK, Lyn, MET, PDGFR, PKA, PKC-α, B-Raf (V599E), SGK-1, Src, VEGFR, and so on, and found that 10% glycerol has a minimal effect on activity in general. We concluded that enzymatic reactions have the same reaction mechanisms as in a large-volume, well-based assay lacking glycerol, as indicated by data consistent for known kinase inhibitor IC_{50}s and K_m for ATP (**Fig. 3-5**).

Tyrosine kinases are key targets in oncology, many of which can be assayed with biotinylated peptide substrates and antibodies against phosphorylated tyrosine residues. For serine/threonine kinases such as B-Raf, antibodies against the phosphorylated substrates are available, as is the case with MEK (**Fig. 5**). For phosphorylated substrates that have no antibodies available for detection, alternative assay formats can be used on the microarray platform. For example, we previously reported the homogeneous PKA assay using the ProFluor™ technology.[11] Heterogeneous-based assays can be performed with either radiolabeled ^{33}P-ATP or specific phosphate-binding fluorescent entities that differentiate phosphorylated peptide from unphosphorylated peptide.

In comparison to binding assay formats, an advantage of activity screening is that both ATP and substrate competitive inhibitors can be screened with a simple change in assay conditions. For example, with excessive substrate and low ATP concentration, ATP competitive inhibitors will be preferentially identified. With excess ATP, detecting inhibition of the enzyme-substrate binding is favored. Most existing kinase inhibitors under investigation target the ATP binding site, which is a nonpolar cavity containing specific hydrogen-bonding groups for recognition of different compounds.[15,30] However, many inhibitors lack complete specificity and target other kinases with similar affinity[15,31] because the ATP binding site is conserved in the human kinome.[1] In situations where ATP-competitive inhibitors may not fully distinguish among kinases with similar ATP binding sites (e.g., the insulin-like growth factor-1 receptor and the insulin receptor), substrate-based inhibitor selection may be advantageous.[32,33] Microarray HTS allows simultaneous kinase screening at varying ATP and substrate levels, along with kinase selectivity profiling, by use of a single print run.

To date, the use of nanoliter reaction microarrays has produced results that have been validated in well-plate assays, including the discovery of a caspase inhibitor[11]; the identification of the proline P2 requirement of human, bovine, and salmon thrombin[34]; the discovery of the S2-S3 subsite that is cooperative in factor Xa[35]; full P2-P3 profiling of numerous serine and cysteine proteases[34,35]; and accurate IC_{50} determination of an aldehyde inhibitor of caspase 6.[11] The application of chemical microarrays for discovery and analysis of kinase inhibitors has the potential to expand the chemical-kinase interactome.

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