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Nanoliter Homogenous Ultra-High Throughput Screening Microarray for Lead Discoveries and IC_{50} Profiling

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Abstract: Microfluidic technologies offer the potential for highly productive and low-cost ultra-high throughput screening and high throughput selectivity profiling. Such technologies need to provide the flexibility of plate-based assays as well as be less expensive to operate. Presented here is a unique microarray system (the Reaction Biology [Malvern, PA] DiscoveryDot™), which runs over 6,000 homogeneous reactions per 1" × 3" microarray using chemical libraries or compound dilutions printed in 1-nl volumes. A simple and rapid piezo-activation method delivers from 30 to 300 pl of biochemical targets and detector chemistries to each reaction. The fluorescent signals are detected and analyzed with conventional microarray scanners and software. The DiscoveryDot platform is highly customizable, and reduces consumption of targets and reaction chemistries by >40-fold and the consumption of compounds by >10,000-fold, compared to 384-well plate assay. We demonstrate here that the DiscoveryDot platform is compatible with conventional large-volume well-based reactions, with a Z' factor of >0.6 for many enzymes, such as the caspase family enzymes, matrix metalloproteinase, serine proteases, kinases, and histone deacetylases. The platform is well equipped for 50% inhibitory concentration (IC_{50}) profiling studies of enzyme inhibitors, with up to 10 dilution conditions of each test compound printed in duplicate, and each microarray chip can generate over 300 IC_{50} measurements against a given target.

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Introduction

THE LARGE-SCALE DNA SEQUENCING of the human genome represents only a starting point in the future of drug discovery. With many possible drug targets discovered every day and new chemical compounds synthesized daily by drug companies, the lead identification has become more dependent upon the enabling technologies of screening and identifying the lead compounds. Despite the huge increase in research and development spending in pharmaceuticals during the past decade, the number of approved drugs as new chemical entities has only slightly increased. The high productivity and low cost of new technologies were greatly needed in every step of the drug discovery process, for example, a rapid, sensitive HTS technique with extreme low cost. To achieve this goal, assay miniaturization with precise, automated analyte delivery has become an industry trend.[1–3] The recent development of microarray and microfluidic technologies has facilitated the development of high-density, low-volume assays. Success stories have been reported of peptide arrays, antibody arrays, and small chemical compound arrays to study enzymatic activities, ligand bindings, and protein–protein interactions.[4–10]

Traditionally, enzyme assays and HTS for enzyme inhibitors are performed in solution-phase, utilizing automated liquid handlers. Microarray technology has re-

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ABBREVIATIONS: AMC, 7-aminomethylcoumarin; CCD, charge-coupled device; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; FRET, fluorescence resonance energy transfer; HDAC, histone deacetylase; IC_{50}, 50% inhibitory concentration; MAC, 4-methylcoumarin-7-amide; MMP, matrix metalloproteinase; PKA, protein kinase A; R110, rhodamine-110.

duced reaction volumes more than a 1,000-fold, from microliters to nanoliters, but the delivery of bioreagents robotically into these micron-size reaction wells is problematic. Thus, most microarray developers have transformed solution-phase screening into immobilized-format (or solid-phase) screening.[4–10] However, problems such as surface chemistry modification, substrate binding specificity, uniformity, and the orientation of molecular attachment have arisen.[4–7] For example, Salisbury *et al.*[7] created a peptide microarray to determine protease substrate specificity, in which peptide libraries linked with a linker were immobilized on the surface of glass. This type of microarray has a few disadvantages, such as the linking process is costly in both time and labor, and only the end-point reaction is detected so that kinetic constants can only be indirectly derived. Schreiber and coworkers developed elegant immobilization methods to fabricate chemical compound microarrays on glass surface and then used them for enzymatic assays, protein–protein interaction, and HTS.[8,9] This technology is marginally useful for screening the millions of small compounds already on the shelves of pharmaceutical, chemical, and biotechnology companies. The inconvenience and expense of immobilization chemistry are demonstrated in most of the existing microarray systems, and the solid-phase reaction and binding systems also have limitation compared to the traditional solution-phase reaction. To solve these issues, a high-density homogeneous microarray system equivalent to the conventional solution-phase assay, known as the DiscoveryDot platform (Reaction Biology Corp., Malvern, PA), has been developed.[11] With this platform, molecules, including small chemical compounds, peptides, and proteins, can be arrayed without pre-immobilization. By doing so, the enzyme reaction can be performed in a more native-like environment, and the proteins will retain their native structure and activity. Each nanoliter-volume microarray dot is microarrayed on the standard microscope slide and acts as an individual reaction center. An aerosol deposition technology delivers a picoliter volume of target material uniformly into each reaction center. Multiaerosol mists with 2 pl average volume merge with each reaction center to initiate the biochemical reaction. Thus, this platform is similar to the conventional well-based format and could be used for enzyme-based protein profiling, enzyme assays, and HTS for drug discoveries.

In the present study, we reported the first applications of this nanoliter chemical microarray in HTS, IC_{50} determination, and compound profiling. We have developed and optimized assay conditions for various enzyme reactions, such as caspases, MMP, serine proteases, kinases, and HDAC, and demonstrated that this microarray-based nanoliter-volume reaction is compatible with conventional large-volume well-based reactions, with a Z' factor[12] of >0.6 for many enzymes. The platform is well equipped for drug IC_{50} determinations; for example, the chip-based IC_{50} values of caspases and HDAC are nearly identical to IC_{50} values determined by equivalent well-plate assays. Thus, this platform has demonstrated its utility as an HTS tool for IC_{50} profiling studies, with the capability of expediting early candidate selection activities.

Materials and Methods

Materials

Purified caspases, peptide substrates with MCA tag, and peptide inhibitors were purchased from BioMol (Plymouth Meeting, PA). The unconverted MCA substrates prior to cleavage have an excitation maximum at 328 nm and emission maximum at 392 nm, while the fully cleaved product (AMC) has an excitation maximum at 346 nm and emission maximum at 441 nm. Caspase substrate with R110 tag were purchased from Molecular Probes (Eugene, OR), and the APO-ONE™ Caspase-3/7 assay kits were from Promega (Madison, WI). The converted substrates have an excitation maximum at 496 nm and emission maximum at 520 nm. Purified recombinant human thrombin was from Enzyme Research Laboratories (South Bend, IN), and substrate (*p*-tosyl-Gly-Pro-Arg)$_2$-R110 was from Molecular Probes. Coagulation Factor Xa was from Haematologic Technologies, Inc. (Essex Junction, VT), and its substrate Z-Pyr-Gly-Arg-MCA was from Peptide International (Louisville, KY). The EnzChek™ Collagenase Assay kit (including collagenase substrate DQ™ gelatin and its inhibitor, 1,10-phenanthroline) was purchased from Molecular Probes. MMPs were purchased from BioMol, and FRET substrates [MMP-2 substrate, Cy3B-PLGLAARK(Cy5Q)-NH, MMP3 substrate, and Ac-RPK(Cy3)PVENvaWRK (Cy5Q)-NH] were from Amersham Biosciences (Piscataway, NJ). The Profluo™ PKA (cyclicAMP-dependent PKA) kit and the tryptase kits were from Promega (testing reagents courtesy of Dr. Richard Somberg and Mr. Andrew Niles). PKA and PKA inhibitor peptide were from Upstate (Waltham, MA). The HDAC assay kit was from BioMol. The small chemical compound library was ordered from Nanosyn (Menlo Park, CA). All general chemicals and supplies were purchased from Fisher Scientific (Pittsburgh, PA). Microarray slides were from Erie Scientific Co. (Portsmouth, NH) and Full Moon BioSystems (Sunnyvale, CA). The aerosol deposition instrument was developed within Reaction Biology Corp. The research and industry-grade nitrogen gas was purchased from BOC Gases (Baltimore, MD), and gas flow regulators, tubing, and syringes were purchased from Cole Parmer Instrument Co. (Vernon Hills, IL) and Small Parts Inc. (Miami Lakes, FL).

Enzymatic assay in 384-well format

Every enzymatic assay performed on the chip was also performed in parallel in 384-well plate based on the manufacturer's instruction, with and without the desired concentration of glycerol. These assays include Profluo PKA kit for PKA assays, MMP2 assay, HDAC assays, and caspase reactions. The general procedures were similar to the caspase assays detailed here: Substrate with designated concentrations was delivered into each well and vortex-mixed, and then 10 U per well of enzyme was added to activate the pro-fluorescence substrate. Reaction buffer contained 10% glycerol in standard caspase reaction based on the manufacturer's suggestion. In caspase 6 kinetic analysis, substrate [$(DEVD)_2$-R110] concentrations ranging from 0 μM to 400 μM were used, and in the inhibitory IC_{50} study, 50 μM substrate was used for all reactions, and inhibitor (DEVD-CHO) concentrations ranged from 0 to 4 mM. Reactions were carried out at 37°C and were read with a Labsystems (Helsinki, Finland) Fluoroskan Ascent FL™ every 2–5 min for 40 time points.

Microarray spotting and aerosol deposition

The chemical compound and/or peptide substrate were first dissolved in DMSO and then diluted to the desired concentration with reaction buffer containing 10–40% of glycerol. The compound solutions were distributed into a 384-well plate (5–30 μl per well) and printed using a stealth pin (SMP7, TeleChem International, Inc., Sunnyvale) with the GeneMachine OmniGrid (Genomic Instrumentation Services, San Carlos, CA) or the Molecular Dynamics GenIII (Amersham Biosciences). The liquid samples containing enzymes or substrates were aerosolized by pumping through the nozzle with a flow rate of 400 nl/s. The fine mists were then directed onto chips after sheathing with carrier nitrogen gas at a flow rate of 3 L/min.

Enzymatic assay on the chip

Enzyme reactions were performed on polylysine-coated slides or plain glass slides (Erie Scientific) with a total of 1 nl (GenIII printing) to 1.6 nl (GeneMachine printing) per reaction. The general procedure for caspase reactions is as following: Substrate with designated concentrations was microarrayed, and then enzyme (10 U/μl) was sprayed on top of each chip. In kinetic analysis of caspase-6, substrate [$(DEVD)_2$-R110] concentrations ranging from 0 μM to 400 μM were used, and in the inhibition study, 50 μM substrate was used for all reactions, and inhibitor (DEVD-CHO) concentrations ranged from 0 to 16 mM. Reactions were carried out at 37°C and were read with a fluorescent microscope. Because of the low detection sensitivity of MCA-based fluorescence, 200 μM Ac-YVAD-AMC, Ac-DEVD-AMC, and Ac-VEID-AMC were used on the chip. Thrombin, MMP, and PKA assays were performed in the same manner except optimal concentrations of substrates and enzymes were first determined.

Scanning of fluorogenic reactions on the chip

The fluorescence intensity detection of AMC and R110 was performed with a Nikon (Melville, NY) E600 fluorescence microscope equipped with an automation stage controller (Proscan, Prior, Webster, NY) and a cooled 12-bit CCD camera with the Chroma (Brattleboro, VT) ultraviolet-enhanced triple band for DAPI/FITC/Texas Red filter sets. Reaction images were assembled together with ImagePro (MediaCybernetics, Silver Spring, MD), and the final array images were analyzed through either ImagePro or ArrayPro (MediaCybernetics). The IC_{50} calculation and plotting were performed with Graphpad (San Diego, CA) Prism. Cy3 and Cy5 fluorescence was detected with the GenePix DNA arrayer (Axon Instruments, Union City, CA).

Chip-based ultra-HTS

For PKA reactions, a small chemical library specially designed for targeting kinases was supplied by Chemical Diversity Laboratories (San Diego). Small amounts of compounds in DMSO were transferred into PKA reaction buffer with 10% glycerol to make a final solution with 100 μM chemical compounds and 0.75 μM R110

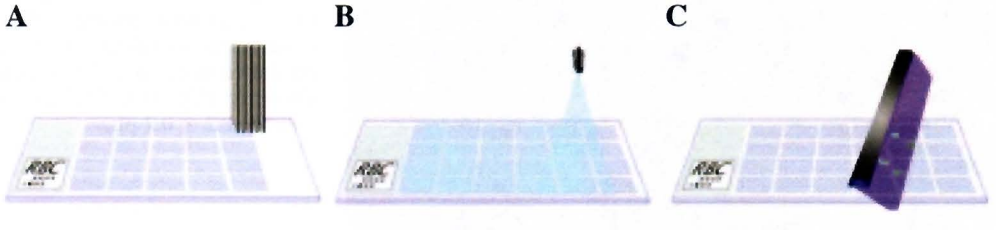

FIG. 1. The execution procedure of the DiscoveryDot platform. **A**: Peptides, proteins, and small molecule compounds are mixed with reaction cocktail and then arrayed onto glass slides as individual reaction centers (RBCs). **B**: The chip is then activated by a fine aerosol mist of biological sample. **C**: Fluorescent signals were detected with imaging instruments such as a laser scanner or fluorescence microscope, and the data were analyzed with microarray software.

substrate. The solution was then arrayed on glass chips and activated with a mixture of PKA (7 U/μl) and ATP (70 μM). After incubation for 3 h at 30°C, the chips were activated again with 5× detection protease.

Chip-based high-throughput IC_{50} profiling

Compounds in DMSO stocks of 1–10 mM were diluted in master-printing 384-well plates in DMSO and then in reaction buffer to obtain 10 μl per well for printing. Each compound was diluted in six doses from micromolar to nanomolar concentration range. Each individual compound was printed with a single pin at six doses in triplicate. The chips were then screened against multiple targets with the corresponding substrates. After incubation, the chips were scanned, the image files were analyzed with the ArrayPro program, and the activity data were normalized and plotted with GraphPad Prism to derive the IC_{50} values.

Results

The DiscoveryDot platform

The mechanism of the DiscoveryDot platform is similar to that of the conventional well-based assay, including library deposition, target delivery, and reaction detection. Chemical compounds were mixed in a cocktail that included 10–40% glycerol for controlling evaporation, 0.1–5% organic solvent (such as DMSO) for compound solubility, and reaction buffer to maintain the biochemical reaction components. The compounds were arrayed on the surface of plain or polylysine-coated slides with a conventional contact pin microarrayer (Fig. 1A). F1 The chips were then activated by spraying the biological target and substrate or detection materials. Aerosol deposition technology converts the biofluid into a fine mist spraying onto the surface of the chip (Fig. 1B). After activation and incubation, the fluorescence signal was detected with a laser microarray scanner or cooled CCD camera-based imager, and interpreted using both imaging and data analysis software (Fig. 1C).

Characterization of the aerosol deposition

Reaction cocktails were arrayed on polylysine-coated slides (Fig. 2A). F2 The arrayed chips were then sprayed with caspase reaction buffer including 100 mM NaCl, 50 mM HEPES (pH 8), 1 mM EDTA, and 100 μM dithiothreitol. Microscopic images (Fig. 2B) demonstrated that repeated spraying with caspase buffer did not significantly alter the morphology of the reaction center. The sprayed mist (5–20 μm in diameter) had a consistent distribution throughout the entire slide. The array design, spot-to-spot spacing, and array size are critical for generating a chip that can be sprayed later with multiple solutions. The characteristics of this specific array were as follows: array spot spacing, 500 μm center to center; array spot diameter, 180 ± 14 μm; array dot volume, 1.6 ± 0.3 nl; spray droplet diameter on chip, 18.1 ± 6.3 μm; spray droplet volume, 2.2 ± 0.2 pl. Successful aerosol delivery to the slide was determined by the optimal op-

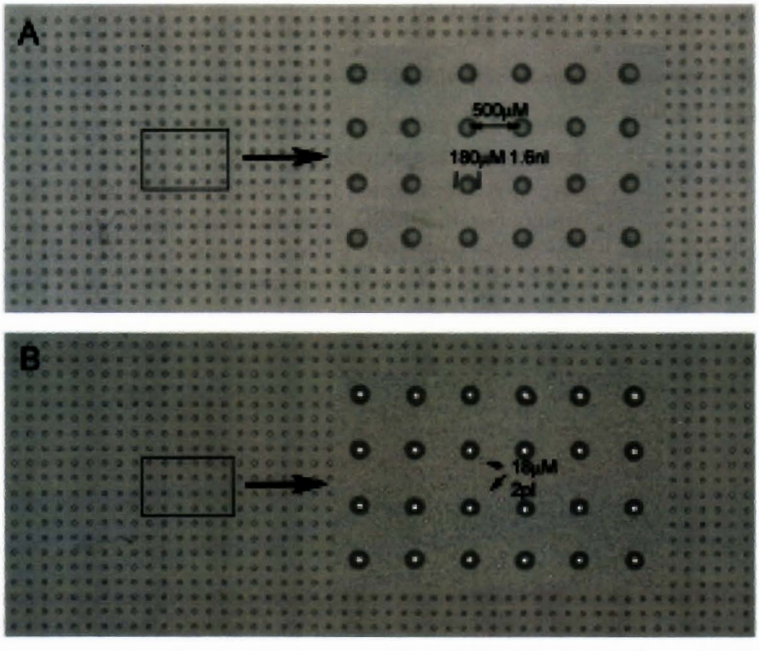

FIG. 2. Demonstration of operational parameters for the DiscoveryDot enzyme chip platform. Small synthetic peptides were dissolved in 10% dimethylsulfoxide and 40% glycerol and arrayed on the surface of glass slides. Pictures were taken before (**A**) and after (**B**) aerosol deposition. The following conditions were used: array spot spacing, 500 μm center to center; array spot diameter, 180 ± 14 μm; array dot volume, 1.6 ± 0.3 nl; spray droplet diameter on chip, 18.1 ± 6.3 μm; spray droplet volume, 2.2 ± 0.2 pL.

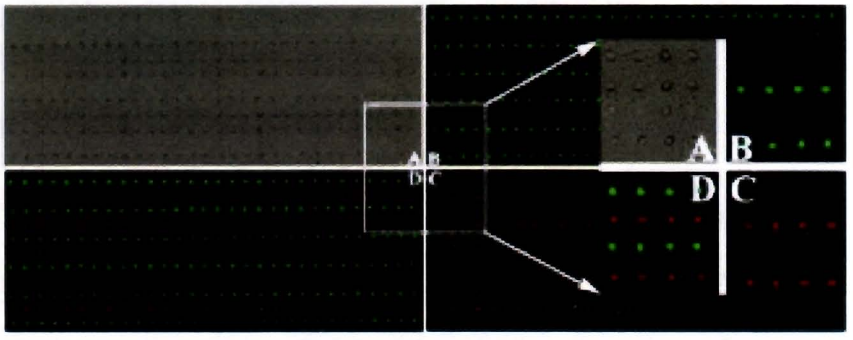

FIG. 3. No-cross reaction among reaction centers after spray. The fluorescein isothiocyanate (green) and rhodamine (red) dyes were arrayed in alternating rows, and the chip was sprayed eight times with caspase reaction buffer. **A**: Morphology of dots after spraying. The fine mist of spray could be observed very clearly in the close-up bright-field view. **B** and **C**: Each fluorescence channel separately. **D**: Combined pictures. The fluorescence values had no change before and after spraying indicating no cross-contamination was detectable.

erational parameters of the spray systems. Through repeated testing, we determined the parameters as follows: biological sample flow rate, 400 nl/s; slide deck velocity, 500–750 rpm.

Because there are no physical boundaries around reaction centers, a critical requirement for this homogeneous array is preventing mixing or bridging of adjacent array spots during aerosol deposition or reaction. We have arrayed both FITC and rhodamine dyes in alternative rows and then sprayed eight times with caspase reaction buffer (Fig. 3). The changes of fluorescence intensity in each row, before and after spray, were within instrumental error, such as <0.01% change for rhodamine and <0.1% change for FITC, demonstrating that there was no cross contamination after repeated sprays.

Validation of enzymatic activities on DiscoveryDot condition

The major difference between this microarray-based nanoliter-volume reaction and the conventional well-based solution-phase reaction was that we used ≥10% glycerol to reduce evaporation and enhance long-term storage. Glycerol is a good protein stabilization material used in daily protein storage, but at a higher concentration, it may affect enzymatic activity because of higher viscosity. In order to investigate this, we carried out several caspase kinetic studies under high glycerol reaction conditions and compared the results with data from the manufacturer (BioMol) (Table 1). Caspases-1, -3, -6, and -8 reacted with their respective specific substrates, Ac-YVADAMC, Ac-DEVD-AMC, Ac-VEID-AMC, and Ac-IETC-AMC. The manufacturer's K_m values for caspases-1, -3, and -6 in 10% glycerol are consistent with the experimental K_m value derived in the 40% glycerol condition. The K_m values in 10% and 40% glycerol were also within a twofold difference for caspase-8 (Table 1). Although many proteases can tolerant high glycerol concentration, we generally maintain its concentration around 10%.

Applications in enzymatic assays

This microarray-based nanoliter-volume reaction platform has wide applications in enzymatic assays. Here, we demonstrated that enzymes like thrombin (a serine protease, a classical drug target for prevention of thrombotic complications), caspases (cysteine protease, important apoptosis indicators), MMP (targets for indications like inflammation, arthritis, emphysema, and multiple stages of tumor initiation, growth, and angiogenesis), and PKA (kinase) are all adaptable on chip (Fig. 4).

Figure 4A demonstrates the thrombin reactions. Reaction cocktail with (top two rows of subarray with 960 reactions) or without (lower one row of subarray with 480 reactions) thrombin substrate at 50 μM was arrayed on

TABLE 1. KINETIC COMPARISON OF CASPASES IN MANUFACTURER'S SUGGESTED REACTION CONDITION WITH HIGH GLYCEROL CONDITION IN 384-WELL PLATES

	Enzyme							
	Caspase-1		*Caspase-3*		*Caspase-6*		*Caspase-8*	
Glycerol	10%	40%	10%	40%	10%	40%	10%	40%
K_m (μM)	14[a]	15.4 ± 1.3	9.7[a]	8.0 ± 0.6	30[a]	23.8 ± 1.3	21.2 ± 2.3	39.4 ± 1.4

[a]Supplied by the substrate and enzyme manufacturer, BioMol.

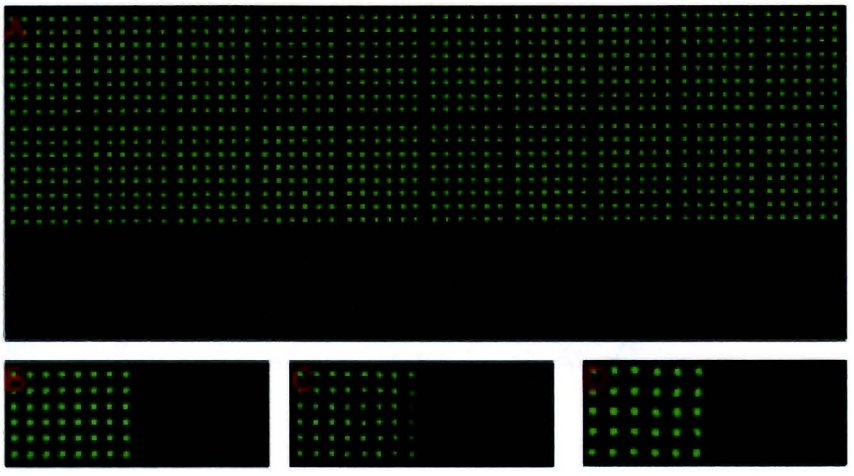

FIG. 4. Examples of the DiscoveryDot chip's application. Panel **A**: A close-up view of the thrombin reaction chip. **B–D**: Small sections of caspase-6, matrix metalloproteinase (MMP), and protein kinase A (PKA) reactions, respectively. Thrombin and caspase chips were arrayed with 50 μM rhodamine-110-based substrate in positive controls and without in negative controls. The MMP chip was arrayed with 0.1 mg/ml DQ gelatin in positive controls and 9 mM inhibitor of 1,10-phenanthroline in negative controls. The PKA chip was arrayed with 5 μM PKA substrate, and 7 μM peptide inhibitor was included in negative controls.

a plain microscope slide. The chips were then activated with thrombin at concentration of 50 U/ml (Fig. 4A). After a 20-min incubation, the chip was scanned and analyzed, and the Z' factor value was 0.7 for the assay.

Figure 4B shows a portion of a caspase-6 reaction (a total of 1,536 reactions). The reaction cocktail with (left subarray) or without (right subarray) substrate (50 μM) was arrayed on polylysine-coated slides and activated with enzyme (10 U/μl). The overall Z' factor after scanning and data analysis was 0.6.

Figure 4C demonstrates the MMP reactions with FRET-based substrate. Reaction cocktail with fluorescein-conjugated DQ gelatin (0.1 mg/ml) with or without inhibitor, 1,10-phenanthroline, was arrayed and activated with collagenase (300 U/ml). The overall reactions' Z' factor was 0.6 (total of 1,536 reactions). We have also adapted the FRET-based MMP2 and 3 assay kits from Amersham Biosciences on chip with good reaction signals (data not shown).

Protein kinases catalyze transfer of the γ-phosphate group of ATP to the hydroxyl groups of serine, threonine, or tyrosine residues in their substrates. Their functions make them valuable drug targets since these phosphorylation reactions play pivotal roles in cellular functions, including cell growth and differentiation, cell shape, and metabolism. To test the DiscoveryDot chip on kinase assay, we have evaluated the ProFluor PKA Assay kit from Promega. A reaction mixture including 1× R110 substrate and 3× PKA in 0.4× reaction buffer with (Fig. 4D, left subarray) and without inhibitor peptide (7 μM, Fig. 4D, right subarray) were arrayed on a polylysine coated slide. The chips were activated by aerosol deposition of 5x ATP, after incubated for 3 h at room temperature the chips were again aerosol deposited with 5× protease came along with the PKA kit. The Z' factor was determined to be >0.6.

Enzyme reactions on chip were dose and time dependent, and the experimental kinetic constant could be derived

Because of the unique solution-phase reaction system, the microarray-based nanoliter reactions were dose dependent and time dependent just as all the well-based large quantity reactions. We have performed comparison studies of kinetic assay for caspase-6 in 384-well plate and in the microarray-based nanoliter reaction platform with R110-labeled substrate, $(DEVD)_2$-R110, in a buffer containing 100 mM NaCl, 50 mM HEPES (pH 8), 1 mM EDTA, and 100 μM dithiothreitol in 25% glycerol. The reactions on chip were performed after different concentrations of substrate were arrayed and activated with enzyme (10 U/μl). Reactions were monitored for hours, and the dose- and time-dependent reactions were plotted (Fig. 5). The K_m value derived from the microarray chip was 20.3 μM and from the 384-well reaction was 30 μM as reported by the manufacturer. Both assays generated similar kinetic constant values. Thus DiscoveryDot was well suited in regular drug screening and enzymatic inhibition studies. For example, when we arrayed different concentrations of inhibitor peptide (DEVD-CHO) with 50 μM substrate of $(DEVD)_2$-R110 and activated by spraying of caspase-6 at a concentration of 10 U/μl, the IC_{50} value was 133 μM (Fig. 6A). The same assay run in parallel in 384-well plates using 10 μl total volume and 50 μM substrates resulted in an IC_{50} value of 140 μM (Fig. 6B). The chip-based results were derived from four repeat assays on two different chips, and each assay point was derived from one subarray of 25 replicate reactions (Fig. 6C). These data demonstrate that both assays yielded the same results, however, the multiple chip assays could yield data in which we have more confidence since the extremely small reactions were cheap and sim-

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F6

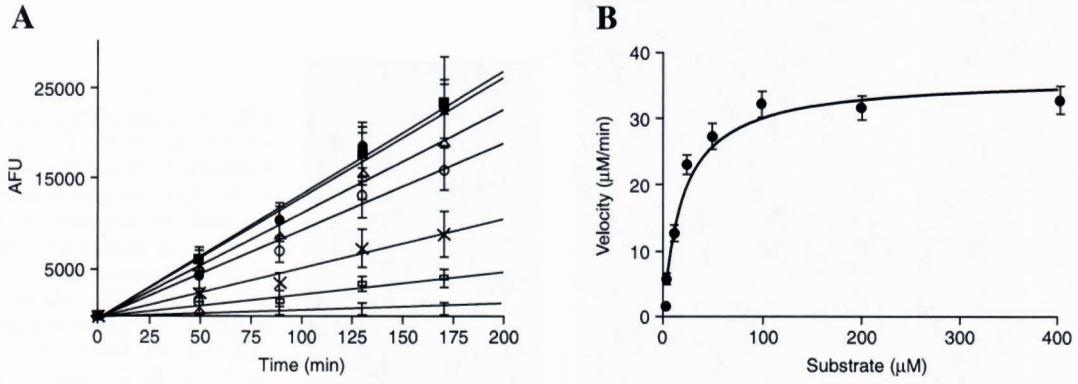


FIG. 5. Time course of caspase-6 reaction on DiscoveryDot and its Michaelis–Menten plot. Caspase-6 reactions were initiated by spraying 10 U/μl caspase-6 over the slides, which had printed reaction centers with different concentrations of substrate, $(DEVD)_2$-rhodamine-110. The fluorescence signals from rhodamine-110 were measured by a microscope-based charge-coupled device camera, and the image obtained was analyzed using ArrayPro software. The substrate concentrations are, from the top, 400, 200, 100, 50, 25, 12.5, 6.25, and 3.13 μM. The reactions are linear up to 3 h (**A**). Velocities were obtained from slopes of A, and the arbitrary fluorescence unit (AFU) value was converted into micromolar units using a standard curve of rhodamine-110 printed on slides and measured the same manner. Kinetic constants obtained from the best fit of nonlinear regression are K_m for substrate = 20.3 μM and V_{max} = 36.2 μM/min (**B**).

ple enough to be repeated many times. The low coefficient of variation values observed at the lower concentrations also demonstrated that the chip assay was very sensitive and well positioned to detect a full spectrum of potential inhibitors. The chip-based inhibition of the HDAC assay was also performed and compared with the manufacturer's inhibition data for a potent HDAC inhibitor, trichostatin A. The IC_{50} value after plotting data from the manufacturer (BioMol) was 3.1 nM, while the value from the microarray-based reaction was 7.4 nM (image and plots not shown). All these experiments demonstrated that the DiscoveryDot platform yielded inhibition data comparable to those of the conventional large-volume assays.

HTS of small chemical compound libraries

One major application of the DiscoveryDot technology is to use it as an ultra-HTS tool for drug discovery. One of the advantages inherent in this platform is that the total cost of DiscoveryDot HTS is only a small fraction of that of conventional HTS because of the nanoliter reaction volume. For example, a 150,000-reactions HTS against Factor Xa was performed on chip (Fig. 7); the total reaction is activated in under 15 min with less than 7.7 U/ml Factor Xa enzyme. The total costs of reagent and materials are less than 0.2¢ per reaction (Table 2). The potency of inhibitions was demonstrated in the amplified microscopic image, which was analyzed with

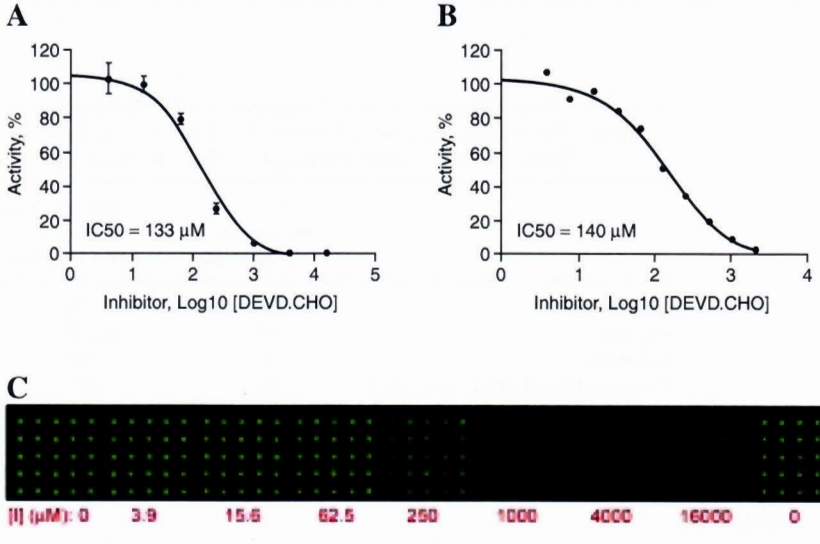


FIG. 6. Inhibition comparison between the DiscoveryDot chip and 384-well reactions. Different concentrations of peptide inhibitor (DEVD-CHO) with 50 μM substrate of $(DEVD)_2$-rhodamine-110 were arrayed on the chip and then activated with spraying of caspase-6. The IC_{50} value derived from the chip was 133 μM (**A**). At the same time the inhibition studies were also performed in 384-well reactions, and the IC_{50} value was 140 μM (**B**). **C**: Sample of DiscoveryDot inhibition reactions.

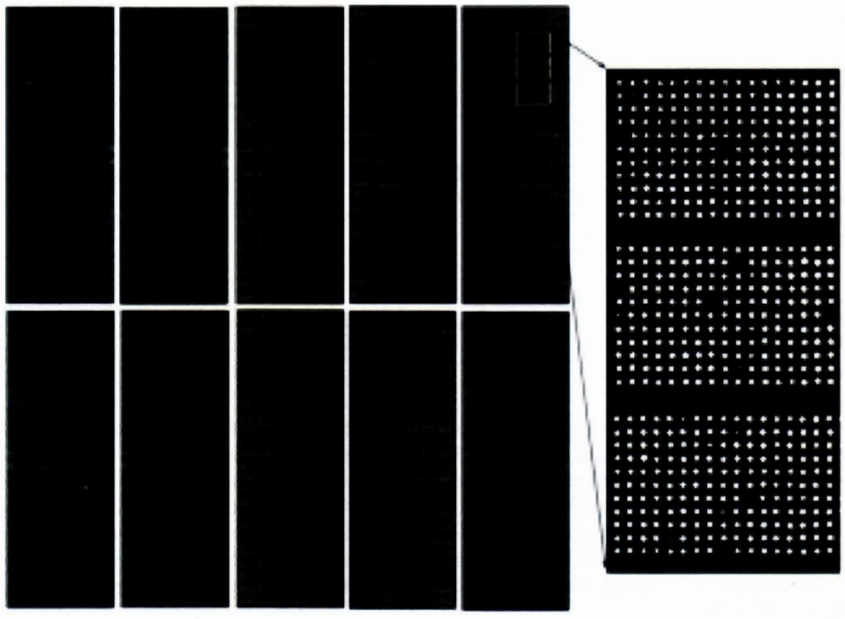


FIG. 7. Ultra-HTS assay for Factor Xa. A library of over 150,000 compounds (10 μM) was arrayed on a chip and screened against Factor Xa with the substrate D-CHA-Gly-Arg-7-aminomethylcoumarin. 3,300 compounds were arrayed in duplicate on each side of the chip with a total of 6,600 reactions on one chip. This image shows 66,000 reactions arrayed on 10 DiscoveryDot chips, which is equal to 178 of 384-well plates. The amplified microscopic image demonstrates the inhibitory effect.

AU6

DNA microarray programs. Another advantage of the microarray is that the microarrayer could produce multiple sets of identical chips from one set of printing plate of the chemical library, while conventional HTS needs each set of chemical library for each target. The multiple replications of these chips can be used for a HTS campaign against a new target at a future date.

AU3

This microarray-based nanoliter-volume technology can also be applied to complicated kinase screening. For example, a small focused kinase focusing library was screened against PKA by using the ProFluo kit. In an attempt to find all potential inhibitors, a high concentration of compounds (100 μM) was arrayed. The results of this experiment uncovered that 6.8% of the compounds in the library displayed over 50% inhibition against PKA (Fig. 8). In this experiment, each compound was arrayed in quadruplet, and a real inhibitor will yield consistent data in each of these reactions; therefore, no secondary confirming HTS is needed.

F8

High-throughput selectivity and IC_{50} profiling against multi-targets

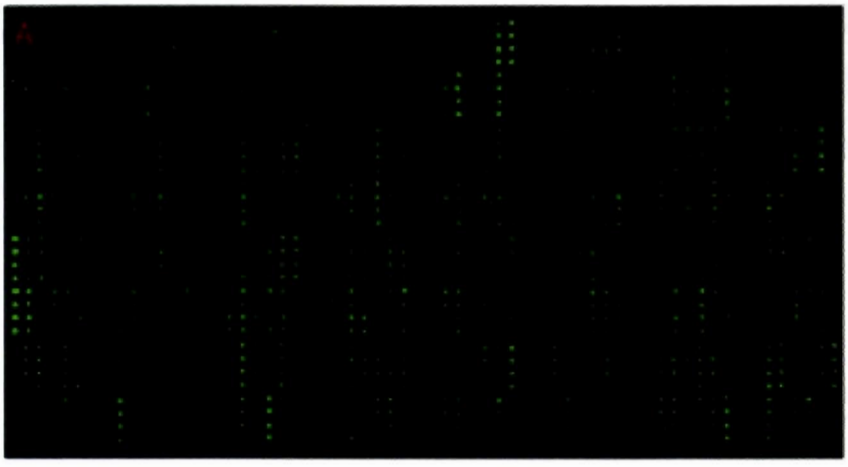
The microarray-based nanoliter-volume platform is a very efficient way to perform high-throughput selectivity and IC_{50} profiling against multiple enzymes of interest. In a proof-of-concept experiment, a group of compounds was arrayed randomly on chip. Each compound had six dose conditions, and each condition was arrayed in triplicate; the first dose in column 1 had no inhibitor as control, the highest inhibitor concentration (100 μM) was in column 2, and then continuously decreased with

TABLE 2. COST COMPARISON FOR SCREENING A 6,144 COMPOUND LIBRARY AGAINST FIVE SERINE PROTEASES, ELASTASE, FACTOR XA, THROMBIN-α, TRYPSIN, AND UROKINASE, FORMATTED INTO ONE MICROARRAY CHIP OR 16 OF 384-WELL PLATES

	Reagent's itemized cost ($)		
	DiscoveryDot	*384-well plate*	*Saving multiple*
Chip/plates	23	120	5
Enzyme	10	3,250	325
Substrate	1	600	600
Consumables (buffer, tips, etc.)	30	875	29
Reagent cost per reaction	0.002	0.158	79
FTE	180	260	1.4
Total cost per reaction	0.008	0.166	21

AU7

FTE,



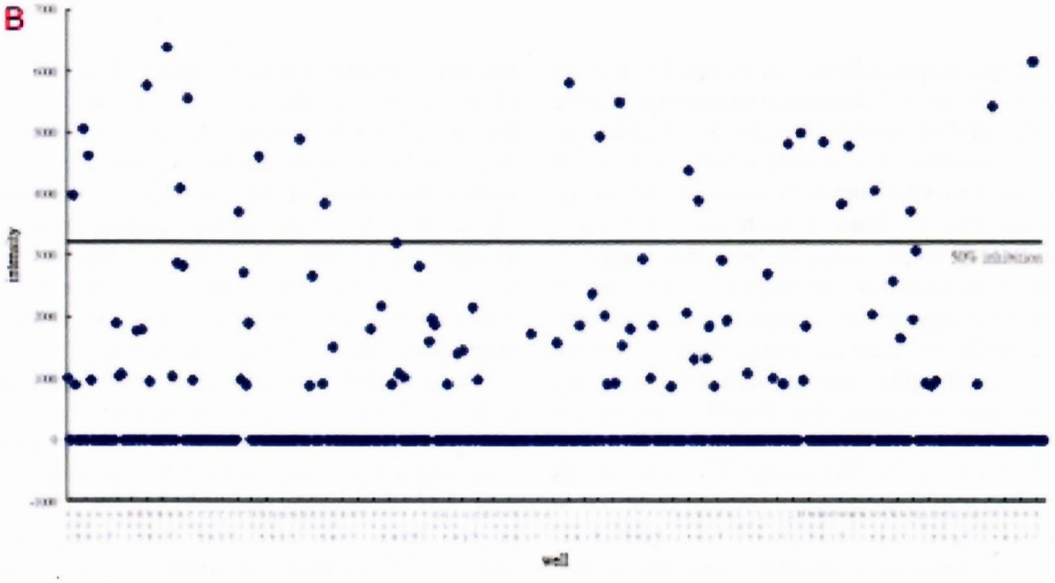


FIG. 8. Ultra-HTS assay for protein kinase A (PKA). Chemical compounds (100 μM) and 0.75 μM substrate were arrayed and activated with a mixture of PKA and ATP. The chips were activated again with protease supplied from the manufacturer, which only cleaves unphosphorylated substrates to yield fluorescence signals (**A**). The primary screening indicated that 6.8% of the compounds demonstrated an inhibition effect of over 50% (**B**).

AU8

log fashion until 10 nM. The chips were then screened against two recombinant human serine proteases, mast cell tryptase and thrombin, and one cysteine protease, recombinant human caspase-6 (Fig. 9). Compounds coded in red inhibited both serine proteases, but IC_{50} values were 43 μM and >1 mM for tryptase and thrombin, respectively. Compounds coded in blue inhibited only tryptase with an IC_{50} of 194 μM, and compounds coded in green had no effect on both serine proteases but had high potency toward caspase-6 with an IC_{50} value of 13 μM.

F9

Discussion

The drug discovery process is critically dependent upon the ability of screening efforts to identify "hits" with therapeutic potential. This screening effort is one of the bottlenecks in the process of drug discovery. In the postgenomic era, against both growing drug targets and combinatorial chemical compounds, the development of extremely low-cost and high-density enzymatic assays is significant. Despite the advance in DNA microarray development, the utilization of protein chips and small chemical compound array chips for drug screening is still a research fantasy.[8,9,13] The existing protein array and small chemical compound array are two technologies that originated from DNA array, and both of them adapted an immobilized format (or separation format). However, a homogeneous-format (or non–separation-format) microarray is very attractive because the assay format is simple, easy, fast, and cost-effective compared to traditional assays. To save precious reagents, miniaturization in HTS has been the trend in the pharmaceutical industry; however, its widespread adoption has been slow since miniaturization has associated high set costs, as well as

FIG. 9. Selectivity and IC_{50} profiling assay. A small selected library was diluted in log fashion, arrayed in multiple sets, and then screened against thrombin, tryptase, and caspase-6. A compound in the red rectangle inhibits both tryptase and thrombin, but is more potent towards tryptase. A compound of in the blue or green rectangle only inhibits tryptase or caspase-6, respectively.

the need for specialized robotic and automation process to support it. The aerosol deposition technology adapted by the DiscoveryDot system provides an efficient and cost-effective solution. It is a simple but versatile tool that can be used in delivering both chemical and biological materials. With a volume of <2 nl for each reaction center, a conventional 384-well plate with an average volume of 50 μl could be used to make a hundred sets of small chemical compound microchips, and each set could be used for a different target screening. With a microliter range of biological fluid to activate each slide containing up to 6,600 reaction centers, this ultra-HTS system can save over 90% of the cost of conventional HTS screening (Fig. 7 and Table 2). The overall Z' factor for this nanoliter-volume microarray platform was from 0.6 to 0.7, which is slightly lower than 0.8–0.9 reported for many 384-well plate assays. However, since the reaction volume is so 16 Ma et al. small, we usually run reactions in duplicate or quadruplicate, so that more confident data in screening will be generated (Fig. 8), which can also eliminate the secondary, confirming HTS performed in conventional methods.

Evaporation is a problem for all miniaturized reaction systems, including the 1,536-well format. To solve this, we have tested many common viscosogens and polymers, such as glycerol, sucrose, polyethylene glycol, etc., and eventually selected glycerol, since it is already well used in enzyme storage, inert to enzyme reactions, and safe. More importantly, glycerol is also freely mixable with reaction buffer and DMSO. However, a high glycerol concentration is known to decrease many enzyme activities. In general, if a major conformational change is involved in enzyme catalysis, this change becomes the rate-limiting step in highly viscous solutions.[14] The decrease in k_{cat} is linearly dependent on solution viscosity.[15] In addition, the diffusion of substrate and product is also affected in highly viscous solutions. On the other hand, it has been reported that the catalytic efficiency (k_{cat}/K_m) of nonreceptor tyrosine kinase, v-fps, for phosphorylation of peptide substrates was not affected by the increase in glycerol concentration while k_{cat} was decreased.[15] We made similar observations: that V_{max} is more affected by glycerol than K_m. Proteases are relatively more tolerable to high glycerol concentration than kinases. In general, we suggest testing the glycerol effect on every enzyme before the reaction, even though 10% glycerol has a minimum effect on most kinases we tested.

In today's 500 total therapeutic drug targets, enzymes make up about 28%,[16] and the number is still growing with post-genomic research. Target such as protein kinases, one of the most important drug target families with over 500 mammalian members known to date and a greater number predicted from genome sequencing and analysis,[17–19] are being evaluated for growth, differentiation, and signaling process involved in tumor progression, cancer, and inflammatory processes. Diseases of cardiovascular, oncological, inflammatory, immunological, and infectious origin may be successfully resolved by interrupting an enzymatic pathway intricately associated with the expressed pathophysiology. Enzyme inhibitors of serine proteases with therapeutic effects include antithrombins for anticoagulation. Inhibitors of human immunodeficiency virus proteases and reverse transcriptases have revolutionized the treatment of acquired immune deficiency syndrome. Metalloprotease inhibitors are under development for the treatment of various cancers. Caspase inhibitors may be the basis for drugs with various disease targets such as cardiovascular disease, stroke, and other degenerative diseases. Tyrosine kinases are involved in immune, endocrine, and nervous system physiology and pathology and thought to be important in the development of many cancers. Many enzymes of interest, including tissue plasminogen activator, kallikrein, plasmin, thrombin, activated protein C, factor Xa, factor

XIa, factor VIIa, peptidases, MMPs, elastases, caspases, furin, cathepsins, trypsin, chymotrypsin, viral proteases, phosphatase, and kinases, already have fluorogenic substrates that can be optimized for analysis. These enzyme screenings could be directly performed on DiscoveryDot chips.

In summary, compared to conventional well-based assays, DiscoveryDot chips drastically reduce the cost of HTS in many areas. First, the nanoliter reaction volume reduces the direct cost for library compounds and drug targets since one compound printing plate can produce hundreds of microarray chips for multiple targets. Second, nanoliter-volume reactions reduce indirect cost in chemical synthesis, compound handling, and target production. Third, nanoliter-volume microarray chips' fabrication only requires a low-cost glass slide and a minimum amount of consumable chemical and bioreagents. By comparison to immobilized format microarray systems, the solution-phase microarray chip also has several advantages. First, each arrayed dot may contain different chemicals and be used as a unique reaction center, so that homogeneous reaction can generate data unobtainable from the immobilized format, including enzyme kinetics. Second, without immobilizing molecules, the solution-phase microarray chip is capable of arraying any existing library chemical compounds for HTS. Third, the DiscoveryDot is a very stable and yet flexible system; molecules like peptides, proteins, and antibodies are all adaptable for protein functional screening and antibody–antigen-based assays. Finally, this platform utilizes all instruments existing in many pharmaceuticals and academic institutions, such as liquid handlers, DNA microarrayers, chip scanners, and data analysis software; therefore it is simple and easy to implement this technology.

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