1

A Homogenous Microarray for Enzymatic Functional Assays
Chemical Compounds Microarray

Haiching Ma*, Yuan Wang, Amy S. Pomaybo, and Connie Tsai
Reaction Biology Corporation, Malvern, PA 19355, USA

Abstract: Microarrays as an emerging research tool promises to play a pivotal role in the post genomic era. However, in spite of the fast development of this technology special requirements, such as the immobilization and delivery of bio-reagents on the chip surface limit the utilization of microarrays, especially for small chemical compound libraries. We have developed a unique homogenous microarray system that overcomes these limitations and can be used to array most biofunctional molecules, such as small chemical compounds, peptides and proteins without pre-immobilization. A standard microscope slide containing up to 5000 microarray dots, with volumes less than 2 nanoliter each and acting as individual reaction centers, can be printed with standard DNA arrayer. An aerosol deposition technology was adapted to deliver extremely small volumes of biofluids uniformly into each reaction center. The fluorescence based reaction signals were then scanned and analyzed with standard DNA scanner and DNA array analyzing software. With this platform, we demonstrated that this chip format could be used for not only screening individual but also multiple enzymatic activities simultaneously with different fluorescent tagged small peptide libraries. We further demonstrated that this system could be a very powerful ultra high throughput screening tool for drug discovery, with which we identified potential "hits" after screening chips printed with small chemical compounds against caspases 1 and 3. This highly sensible chip is also able to monitor caspase protein expression profiles by activating the peptide chips with cell lysates undergoing apoptosis.

Key words: Small chemical compounds microarray, peptide array, enzymatic assay, aerosol deposition, ultra high-throughput screening.

Introduction

The large scale DNA sequencing of the human genome represents only a starting point in the future of biology. New demands are emerging in proteomics – the study of proteins, their expression, and their function. Proteins

are more complex, richer in information, and more relevant to biomedical research than genes. Genomes are essentially fixed for a lifetime; proteomes never stop changing. Protein profiles yield the most clues about the functions of cells and the activity of biological systems. Microarrays, such as peptide arrays, antibody arrays and small chemical compound arrays, have been developed in recent years to study protein function, such as, enzymatic activity, receptor ligand binding and protein-protein interaction (1-6). Traditionally, protein assays and small chemical compound screening are performed in solution-phase utilizing automatic liquid handling machines. Microarray technology has reduced reaction volumes more than a thousand fold from microliters to nanoliters, but the delivery of bioreagents robotically into these micron size reaction centers is problematic. The popularity of microarray technology has transformed traditional solution-phase screening into immobilized-format (or separation-format) screening (1-3). However, problems such as microarray surface modification, substrate binding specificity, uniformity and the orientation of molecular attachment have arisen (1-3). For example, Schreiber and co-workers immobilized newly synthesized small chemical compounds on a chip and used them for enzymatic assays, protein-protein interaction and high throughput screening (4,5). This immobilization technology is marginally useful for screening the millions of small compounds already on the shelves of pharmaceutical, chemical and biotechnology companies. It is cumbersome to immobilize libraries of this size on chips for high-throughput screening. The inconvenience and expensive cost of immobilization chemistry is also demonstrated in other microarray systems, such as the peptide array for kinase reaction (3). To solve this issue, a homogeneous microarray assay system mimicking the conventional protein assay scheme is needed. The major obstacle is how to deliver the biofluid onto the chip without cross-contaminating the individual reaction centers which are less than 2 nl in volume, and approximatly 300 microns apart, while simultaneously avoiding the problems of evaporation inherent to these nanoliter volumes.

Here we describe the first *H*omogeneous *M*ulti-functional *M*icroarray (HMM) system that can be used for multi-screening applications. With this platform, molecules, including small chemical compounds, peptides and proteins, can be arrayed without pre-immobilization. Chips containing up to 5000 dots, less than 2 nanoliter each, can be arrayed on a standard microscope slide with each dot acting as an individual reaction center. An aerosol deposition technology delivers a miniature volume of target material uniformly into each reaction center. By doing so, multi-aerosol mists with 2 picoliter average volume merge with each array dot to initiate the chemical reaction. In this study, we demonstrate that this platform is a versatile tool for multi-functional ultra High Throughput Screening, for enzyme substrate specificity, enzyme activity and antigen-antibody binding assays. We further demonstrate that by screening a small chemical compound library against caspases 1 and 3, in our microarray platform that potential 'hits' can be

identified. By using a caspase substrate array created with this platform, we also show that our chip format is very sensitive for cell lysate screening and for monitoring caspase expression changes through a functional assay during apoptosis. This new functional proteomic assay system has great potential for studies including cell function, drug interaction assays and signaling pathway monitoring.

The HMM Platform and Applications

Overview of HMM system. Miniaturization is a key concept of current microarray technology for high throughput screening to meet the future needs of fast and cost-effective drug discovery, and for diagnostic screening to get more information with small amount available biomaterials. However, many physical problems associated with the producing homogeneous nanoliter biomolecule droplets on a solid surface, such as the microscope slides or chip. For example, nanoliter droplets of biomaterials will dry up within minutes on the surface of the glass slid, and are easily cross contaminated if additional solution added later on. With the size of the array dots, it requires a very prissily control arrayer to re-array each dot to delivery any new materials that needed. The strategy that HMM adapted is to use viscous inactive solvent to prevent the evaporation and cross contamination on the array, and then deliver any required biomaterials through an aerosol deposition system that we were licensed from the University of Pennsylvania. The general procedure is as follow: Compounds were mixed in a cocktail that includes 25-50% of a glycerol-like material for controlling evaporation, 1-10% of an organic solvent, such as DMSO, to enhance compound solubility, and 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 arrayer (Figure 1.1A). The chips were then activated by spraying the screening target material. We adapted an aerosol deposition technology that converts the biofluid into a fine mist for uniform spraying onto the surface of the chip (Figure 1.1B). After activation and incubation, the fluorescence signal was detected with a fluorescent microscope equipped with a cooled CCD camera, and interpreted using both imaging and data analysis software (Figure 1.1C).

Characterization of the Aerosol Deposition. Reaction cocktails (containing 40% glycerol, 10% DMSO and various small synthetic peptides or chemical compounds) were arrayed on the surface of glass slides with a GeneMachine OmniGrid (Figure 1.2A and insert). The arrayed chips were then sprayed with solutions such as water, DMSO, 1% glycerol in water, or caspase 3 enzyme reaction buffer (100 mM NaCl, 50mM HEPES 1mM EDTA and 100 mM DTT). Brightfield images (Figure 1.2B and insert) demonstrated that

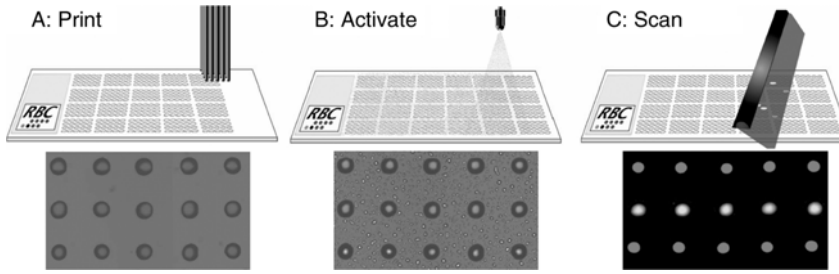

FIGURE 1.1. **HMM system.** (A) Peptides, proteins or small molecules are mixed with reaction cocktail and then arrayed onto glass slides as individual reaction centers. (B) The chip is then activated by a fine aerosol mist of biological sample; the mist droplets fuse with each array dot without causing cross-contamination between reaction centers. (C) Fluorescent signals were detected with imaging instruments such as a fluorescence microscope, and the data was analyzed with microarray software.

repeated spraying with reaction buffer did not significantly alter the reaction center morphology. The sprayed mist (5 to 20 mm in diameter) had a consistent distribution within each slide and throughout the entire slide tray that contained 20 slides. For instance, the actual counted number of droplets on slide areas 1, 2 and 3 (Figure 1.2B) had an average CV of 11% between slides and 7% within slides.

The array design, spot to spot spacing, and array size are critical for generating a protein chip that can be sprayed later with multiple solutions and stored for longer periods of time. The characteristics of this array were: Array spot spacing: 500 um center to center; Array spot diameter: 180 ± 14 um; Array dot volume: 1.6 ± 0.3 nl; Spray droplet diameter on chip: 18.1 ± 6.3 um; Spray droplet volume: 2.2 ± 0.2 pL. Successful aerosol delivery to the slide was determined by the optimal operational parameters of the spray systems. Determined by repeated testing, we set our parameters as follows in most situations: biological sample flow rate 800 nL/s; slide deck velocity: 2.54 cm/s. Distance of nozzle to the slide: 2.54 cm; Nozzle orifice diameter: 0.09 inches.

Another critical requirement for this homogenous array environment is that no adjacent array spots on the slide mix or cross-contaminate other spots during the spray activation of the chip. To illustrate the absence of cross-contamination, we arrayed alternating rows of FITC and rhodamine on chips with a spacing of 500 μm. Multiple sprays (up to 8 mist applications of caspase 3 reaction buffer) did not cause a single example of cross-contamination between the rows or columns for over one thousand spots. The fluorescence signal changes after spraying were minimal, for rhodamine the

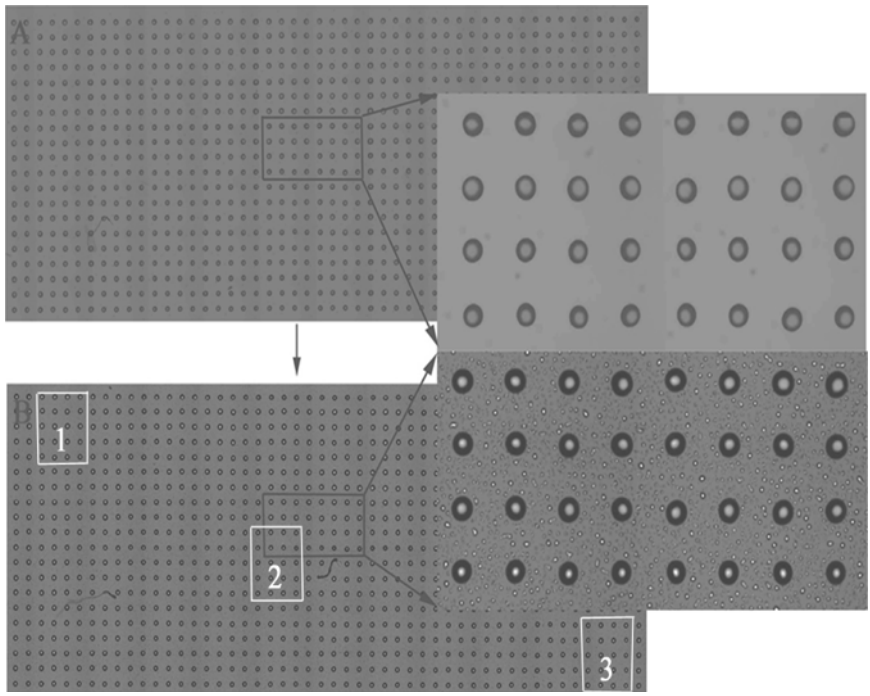

FIGURE 1.2. **Demonstration of operational parameters for the Morewood enzyme chip platform.** Small synthetic peptides were dissolved in 10% DMSO and 40% glycerol and arrayed on the surface of glass slides. Pictures were taken before (A) and after (B) aerosol deposition. The whole array pictures show that array spot morphology was uniform across the slide after multiple sprays with caspase reaction buffer. The enlarged pictures show the close up views of pictures A and B. The mist is quite evenly distributed among the dots, and the array dots are well preserved after multiple sprays. The spray mist distribution (droplets per unit area) was quite uniform; the average mist droplets were very consistent through out the 3 regions (B). The inter-slide CVs of region 1, region 2 and region 3 are 16%, 14% and 3% respectively and the intra-slide CVs of slide 1, slide 2 and slide 3 are 1%, 4% and 16%.

change was <0.01%, and for FITC the change was <0.1% (Figure 1.3). This experiment was repeated with each of the spray solutions mentioned above with the same or similar results.

Validation of Enzymatic Activities in HMM System. The major difference between HMM reaction conditions from conventional solution phase reactions was that we used a higher concentration of glycerol-like material to reduce evaporation and enhance long term storage. Glycerol was a good protein stabilization material used in daily protein storage, but at a high concentration, it may also affect enzymatic activity or antibody-antigen binding characteristics. To investigate this, we carried out several enzyme kinetic

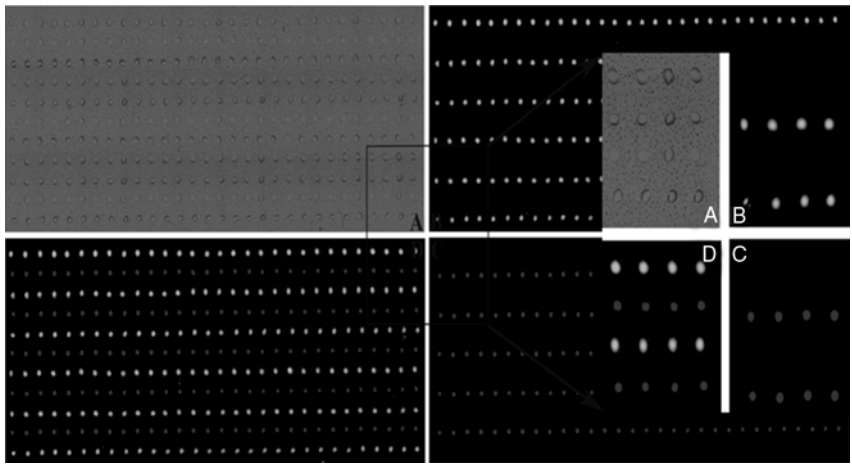

FIGURE 1.3. **Demonstration of no-cross reaction among reaction centers after spray.** The FITC (green) and rhodamine (red) dyes were arrayed in alternating rows and the chip was repeatedly sprayed (8 times) using a caspase 3 reaction buffer. Panel A showed the morphology of dots after spray, the fine mist of spray could be observed very clearly in the close up bright field view. Panel B and C showed each fluorescence channel separately and panel D are the combined pictures. The fluorescence value of FITC dots is roughly equal to the background value when the rhodamine signal was collected, and vice versa. No red-green co-mixing, indicative of cross-contamination was detected after this exposure to 8 separate sprayings of the chip.

studies and antibody-antigen binding assays under 40% glycerol reaction conditions and compared the results with standard reaction conditions. For enzymatic reactions, we used Caspases 1, 3 and 6 with their specific substrates, S1 (Ac-YVAD-AMC); S3 (Ac-DEVD-AMC) and S6 (Ac-VEID-AMC), respectively. The experimental K_m values for caspases 1, 3 and 6 under the 40% glycerol conditions were 13.2 mM, 7.5 mM and 30.1 mM for S1, S3 and S6, respectively, compared to published K_m values from the substrate manufacturer (BioMol) of 14 mM, 9.7 mM and 30 mM in 10% glycerol. The specific activity of these enzymes is 20 to 30% lower in 40% glycerol than in 10% glycerol, but with a longer reaction time, the total substrate conversion could reach the same level (data not shown)

Applications in Single Enzymatic Assay. Caspase cross activity on other caspases' substrates was the first application tested in the HMM system. We arrayed a number of different caspase substrates and activated them with an aerosol of purified caspases. Figure 1.4A showed that caspase 1 not only had enzymatic activity on S1 (subarray 1), but also had cross-reactivity with both S3 (subarray 2), S6 (subarray 3), which was confirmed during a conventional 384-well format experiment (Figure 1.4B).

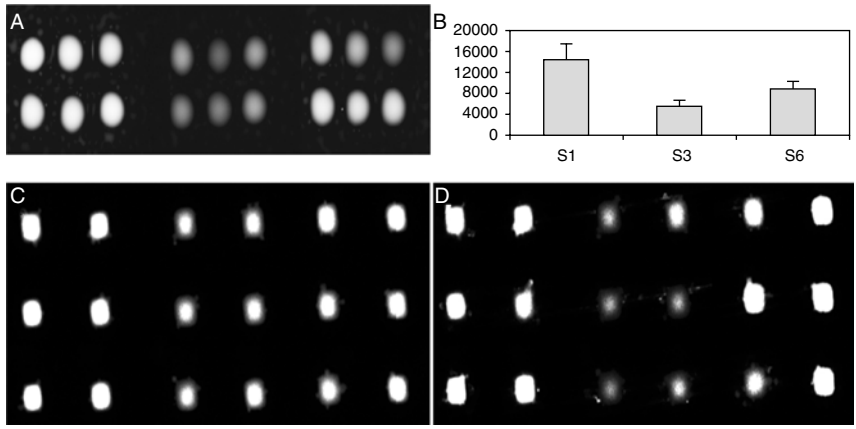

FIGURE 1.4. **Single enzymatic detection.** Substrates for caspases 1, 3 and 6 were arrayed and activated by caspase 1 (A). The cross activities between caspase 1 with substrates of caspase 3 and 6 were well reflected in 384-well reaction, showed in (B). The fluorescently tagged PKA peptide substrate from the IQ™ assay kit was arrayed (C). The left subarray has substrate, inhibitor (50 μM) and PKA. The middle subarray has no inhibitor and the right subarray has no PKA. ATP was sprayed to activate the kinase reaction, and quencher solution was sprayed several hours later to detect the reaction results (D), and only the central subarray showed the desired quench effect.

The HMM system can also be adapted for other commercial available homogenous assays, such as the Kinase reaction illustrated in Figure 1.4C. We arrayed the IQ™ PKA Assay reagents with or without PKA inhibitors on chips, and activated the reaction by spraying ATP. The PKA substrate in IQ™ PKA Assay Kit is fluorescence labeled (Figure 1.4C), and the fluorescence signal will be quenched upon the addition of a phosphate during kinase reaction (Figure 1.4D). Our experiment showed that only the middle subarray had a fluorescence quenching effect caused by the phosphorylation reaction followed by quencher binding. The data clearly demonstrated that the HMM system was capable of differentiating this kinase reaction with or without inhibitors. This application without surface immobilization or radioisotopes is clearly more favorable than other similar microarray approaches (3).

These data demonstrated that utilizing such a peptide array, HMM can be used for screening substrate libraries of single or multiplexed enzymes to search for the best specific substrate, even for enzymes belonging to the same family and having cross-reactivity with other family members. Similarly, we arrayed different enzymes on the chips and sprayed with a single substrate. We were able to detect the activity of each enzyme towards the sprayed substrate (data not shown). This application could be used by manufacturers that are looking for the best enzyme to convert certain substrates.

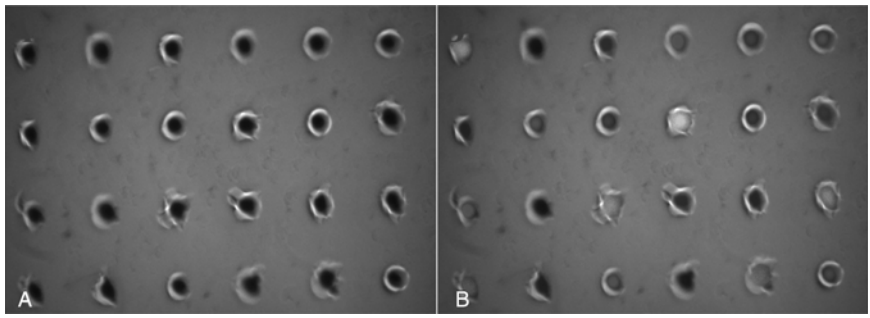

FIGURE 1.5. **Demonstration of multiplex detections.** Thrombin substrate with a blue fluorescent tag and chymotrypsin substrates with a red or green fluorescence tags were arrayed on the chip (A) and activated with thrombin and chymotrypsin simultaneously (B).

Application in Multi-Enzymatic Assay. We next tested the feasibility of multiplexing assays using the HMM system. We have randomly arrayed one thrombin peptide substrate (carbobenzoxy-VPR-MCA) two different chymotrypsin quenching substrates, BODIPY FL and BODIPY TR-X on a chip, then activated by spraying both thrombin and chymotrypsin simultaneously or sequentially (Figure 1.5). The results showed this peptide array could detect both enzymes' activities and the three specific substrates without mixing the signals. So that theoretically, a small chemical compound array in HMM system could be used for screening multi-enzymes activities, as long as the specific activities of enzymes could be differentiated with different fluorescence channels.

High-Throughput Screening of Small Chemical Compound Libraries. One major potential application of the HMM system is to use it as an ultra high-throughput screening tool for drug discovery. With an arrayer producing multiple sets of identical chips from the same small chemical compound library, we believe that each of these chip sets can be used for a single target screening. To establish this concept, we selected caspases as targets. Apoptosis is a genetically programmed, morphologically distinct form of cell death that can be triggered by a variety of physiological and pathological stimuli. The enzyme family of caspases plays a critical role in the initiation and execution of this process. Thus, various pathways in apoptosis are targets of pharmaceutical discovery.

We arrayed multiple identical sets of chips with a library of 380 small chemical compounds (Figure 1.6). On the same chip, a subarray of glycerol dots (A01, row A and column 1) without chemical compound were used as a negative control to show the uninhibited enzymatic activity and two subarrays with known peptide inhibitors of caspase 1 (B01) and 3 (C01) were

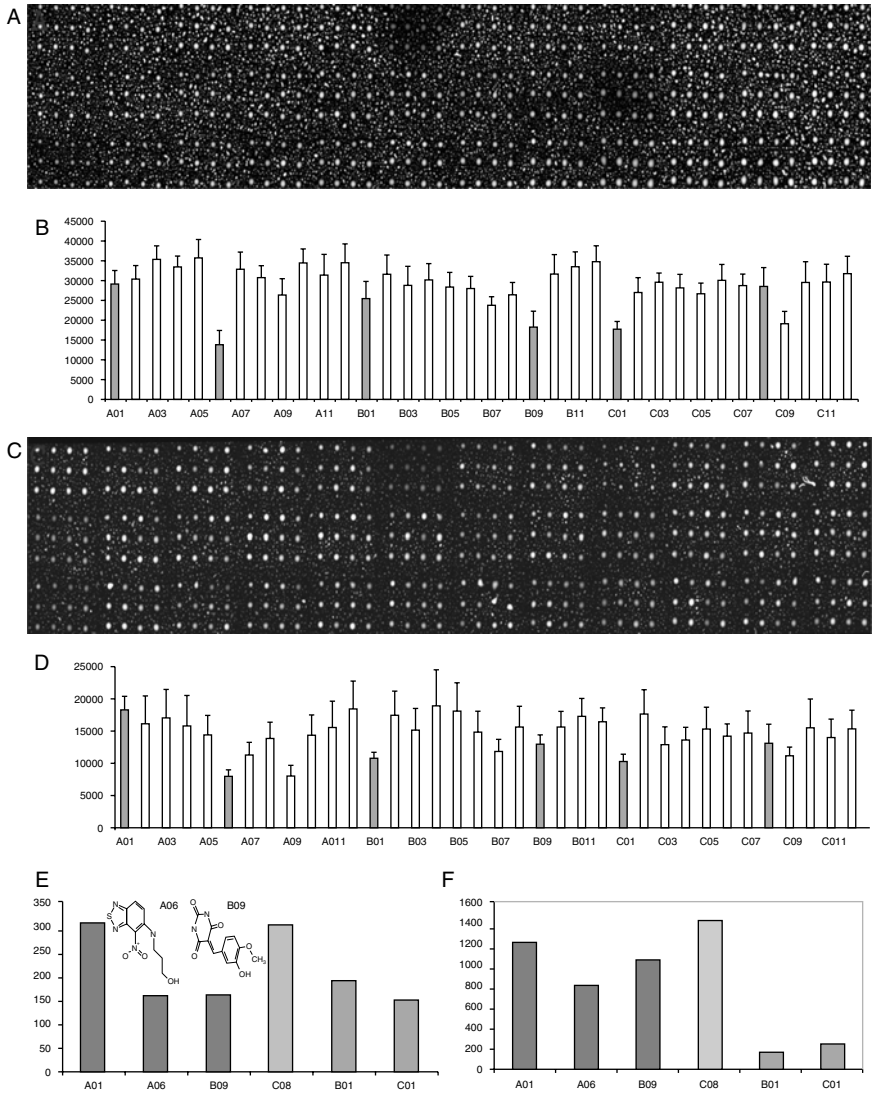

FIGURE 1.6. An ultra high-throughput screening assay with the HMM system. A library of small chemical compounds were arrayed on a chip and screened with caspase 3 (A and B) and caspase 1 (C and D) respectively. Subarray A01 (Blue bar) is a negative control and subarrays B01 and C01 (red bars) are positive controls in each chip (see text for details). Compounds A06 and B09 (Black bars) showed inhibitory effects on caspase 3 compared to the positive control based on image (A) and chip data analysis (B), but only A06 showed a similar effect on caspase 1 (C and D). The conventional 384-well format reactions confirmed these finding for caspase 3 (E) and caspase 1 (F). Compound C08 (Green bar) was included in both assays as an internal control (A-D).

used as positive controls to show inhibited activity (Figure 1.6A and 1.6C). The chips were then sprayed with caspase 1 and caspase 3 in two separate sets, and followed with a second spray of their specific substrates. After an overnight incubation, the slides were scanned on a fluorescent microscope-based scanner to detect potential 'hits' (Figure 1.6A and 1.6C). In this study, we found two potential inhibitors for caspase 3 (Figure 1.6A and 1.6B) and one for Caspase 1 (Figure 1.6C and 1.6D). We then repeated the same reactions in a 384-well format including the known inhibitors, identified inhibitors and a randomly selected compound (C08). This experiment confirmed our microarray findings with caspase 3 (Figure 1.6E) and caspase 1 (Figure 1.6F). The caspases have 4 binding subsites, S1 to S4, and previous researchers have indicated that binding at the S1 site confers the selectivity of caspases, while binding to the S3 and S4 subsites is believed to be critical for differentiating between caspases (7). By comparing the x-ray crystal structure of caspase 3 bound with an isatin sulfonamide inhibitor (8) with these two compounds, the benzothiadiazole ring of A06 has the potential to bind the S2 subsite of caspases, and the ketone carbonyl group of the general barbiturate ring in compound B09 may form a tetrahedral intermediate with the catalytic residue Cys163, and the benzene ring could occupy the S3 subsite. This experiment demonstrated the high-throughput screening capability of the HMM system and identified a compound with both S2 and S3 binding capability which could be further refined by screening a larger library containing additional structural variation (8,9).

HMM Used for Monitoring Apoptosis Process. The amplification techniques for RNA and DNA made the DNA array chip possible for evaluating the gene expression profiles of a given cell type. However, there is no equivalent technique for protein amplification, and it is also difficult to adapt a protein array to evaluate all protein expression profiles because of the complexity of the protein expression pattern and the protein levels at different stages. Antibody arrays have been used to screen protein expression changes (10), but the simple binding assay is not as useful as a functional bioassay. Based on the sensitivity of the fluorescent detection adapted for the HMM system, we believe that the HMM system could be used for detection of protein expression profiles by using a functional enzyme assay. Caspases afford an excellent opportunity for testing this hypothesis. By scanning a 60-compound fluorogenic, positional scanning library of Ac-X-X-X-Asp-AMC, Thornberry (11) has assigned each caspase a specific peptide substrate that has been used to evaluate caspase activity (12). To establish the concept that the HMM system is sensitive enough to be used for cell lysate screening, we compared 3 common caspase activities in Jurkat cells before (Figure 1.7A) and after (Figure 1.7B) camptothecin treatment. In this experiment, we arrayed 4 peptide substrates S1, S3, S6 and S6/8 [a substrate (Ac-IETC-AMC) that can be used for detecting both caspases 6 and 8] and then sprayed the arrays with Jurkat cell lysate. By comparing the fluorescent signals, we found

that each substrate's turnover had increased significantly after camptothecin induction; with substrates 3 and 6 having the highest activity (Figure 1.7C). This assay indicated that the HMM chip is sensitive enough to develop a protein expression profile. Caspases, in general, have high cross-reactivity to the various substrates and inhibitors (11-13). Based on our initial screening assay (data not shown), building a substrate array for separating activities of caspases 1, 3 and 6, requires a minimum of these 4 substrates. To build an assay chip to cover the full spectrum of caspases to monitor the apoptosis process with cell lysates (other enzymes may also have activities on these substrates), an array may have to include all the caspase substrates and combinations of inhibitors (14). Such a chip will have a significant impact on basic apoptosis research, caspase drug development and drug-drug interaction studies.

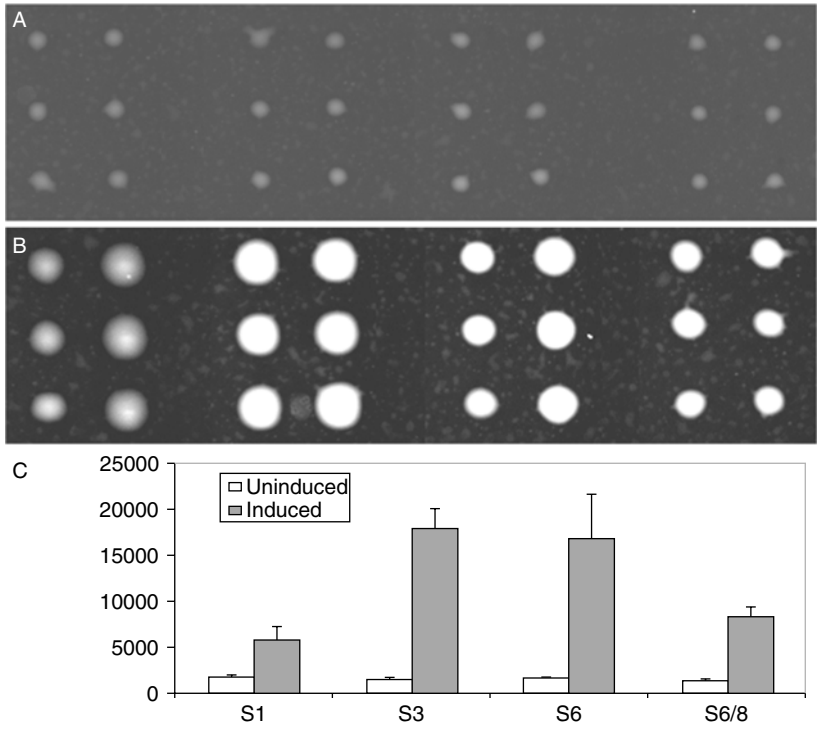

FIGURE 1.7. **Monitoring apoptosis pathways with whole cell lysates.** Substrates of caspases 1, 3, 6 and 6/8 were arrayed and activated with Jurkat cell lysate before (A) and after (B) camptothecin induction. Campothecin induction increased caspase activities 3-9 fold (C).

Conclusion

Despite the great advance in DNA microarray development, the protein chips and small chemical compound array chip are still a researcher or drug discoverer's fantasy (4,5,15). Existing protein array and small chemical compound array are two technologies that originated from DNA array and both of them adapted its immobilized-format (or separation-format). However, a homogenous format (or non-separation-format) microarray will be very attractive because it is simple, easy and fast. It is also very cost effective comparing to traditional assay. For example, the 1536-well with 5-10 μl reaction could save the cost dramatically comparing to the traditional 100-200 μl reaction in 96-well. However, further reducing these reactions to microarray format with nanoliter volume, many physical obstacles exist, and one of the most challenging one is how to precisely deliver sub-nanoliter volume of biomaterials into each reaction center. The aerosol deposition technology that HMM system adapted could be a perfect solution. It offers a versatile assay tool which can control the delivery of inhibitors, substrates, cofactors, or enzymes to each reaction center. With an average volume of <2 nl for each reaction center, a conventional 384-well plate with an average volume of 20 μl could be used to make a hundred sets of small chemical compound microchips, and then could be used for one hundred different target screenings. With an estimate of 3 μl of biological fluid to activate each slide containing up to 5000 reaction centers, the HMM ultra high-throughput screening system can save over 90% of the cost of drug discovery screening.

The functional proteomic activity of HMM could play an important role both in drug discovery and basic proteomic research, since enzymes represent about 28% of current drug targets. Many enzymes of interest, including tPA, kallikrein, plasmin, thrombin (16,17), activated protein C (18), factor Xa, factor XIa, factor VIIa (19), peptidases, matrix metalloproteinases, elastases, caspases, furin, cathepsins, trypsin, chymotrypsin (20), and viral proteases (21) already have fluorogenic substrates that can be optimized for analysis. In addition, quenched fluorescent peptides have been synthesized with phosphotyrosine which can be removed by a phosphatase to reveal a protease sensitive sequence whose cleavage results in a fluorogenic signal (22). Conversely, phosphorylation of a recognition site by a kinase would prevent cleavage by a protease, thus allowing a wide range of microarray-based studies of intracellular signaling enzymes. The drug discovery process is critically dependent upon the ability of screening efforts to identify "hits" with therapeutic potential and the screening efforts is one of the bottlenecks in the process of drug discovery. As an uHTS tool, HMM platform could save both time and money for drug discovery. HMM reduces the direct drug screening cost simply by requiring thousand fold less library compounds and drug targets. It also reduces the indirect cost, such as chemical synthesis and handling. In HMM

system, a traditional small diversified library can be produced into hundreds sets and each of them for different target.

In summary, comparing to immobilized format array systems, the HMM system has several advantages. First, each arrayed dot may contain different chemicals and be used as a unique reaction center. The homogeneous reaction can generate data unobtainable from the immobilized format including enzyme kinetics. Second, HMM eliminates the need to immobilize the arrayed molecules, which allows the arraying of any existing library of peptides or small chemical compounds on chips for high throughput screening. Third, HMM conditions are very stable and yet flexible, and can also be used for arraying proteins and antibodies, which can be used for protein functional screening and antibody-antigen based assays. Fourth, HMM utilizes an aerosol deposition technology that converts a minimum amount of biological fluid into an extremely fine mist and then uniformly deposits it on the chip to activate each reaction center.

Materials and Methods

Materials

Purified caspases, peptide substrates and fluorescent dyes were purchased from BioMol (Plymouth Meeting, PA). The small chemical compound library was ordered from Nanosyn (Menlo Park, CA). Thrombin was from Enzyme Research Laboratories (South Bend, IN), and its fluorescent substrate was purchased from Peninsula Laboratories, Inc. (San Carlos, CA). The EnzChek Protease Assay Kits (both Green and Red) for the chymotrypsin quenching assay were purchased from Molecular Probes (Eugene, OR). The IQ™ Kinase Kit and PKA were purchased from Pierce Biotechnologies (Rockford, IL). The AlphaScreen™ Kit for antibody antigen detection was purchased from PerkinElmer (Meriden, CT). Caspase induced and un-induced cell lysates were purchased from Geno Technology (St. Louis, MO). The research grade nitrogen gas was purchased from BOC Gases (Baltimore, MD), gas flow regulators, tubing and syringes were purchased from Cole Parmer Instrument Company (Vernon Hills, IL). All glass slides, general chemicals and supplies were purchased from Fisher Scientific (Pittsburgh, PA).

Arraying and Aerosol Deposition

The small chemical compounds or peptides were dissolved in DMSO, then mixed with reaction buffer, including 25-40% glycerol as indicated in the text, in 96-well or 384-well plates, and then arrayed with a GeneMachine OmniGrid or stored at –20 to –80 ˚C for later use. The arrayed slides were then activated by aerosol mists of enzymes, cell lysates, or other fluids.

Fluorescence Detection and Data Analysis

The fluorescently tagged substrates were scanned with a fluorescence microscope (Nikon E600) equipped with a cooled CCD camera, X-Y automatic control stage and picture stitching program ImagePro (MediaCybernetics, Silver Spring, MD). The final array picture was then analyzed through a DNA array program, ArrayPro (MediaCybernetics, Silver Spring, MD), and fluorescence intensities were automatically assigned.

The Determination of K_m Values of Caspases at High Glycerol Concentration

Reactions were performed in a 384-well plate, and in each 10 µl reaction, 20 U of enzyme was added to activate the pro-fluorescence substrate. Reaction buffer contained 40% glycerol, and the substrate concentrations ranged from 10 µM to 160 µM. Reactions were carried out at 37 ˚C and were read with a Labsystems Fluoroskan Ascent FL every 2 to 5 minutes for 40 time points.

Peptide and Small Chemical Compound Chips

Peptide substrate concentrations were 200 µM for caspases 1, 3 and 6, 500 µM for thrombin and chymotrypsin. The concentration of small chemical compounds on the chips for caspase 1 and 3 screening was 300 µM. Purified caspases used for spraying were 10 Unit/µl with 3µl/spray/slide on the average. The induced and uninduced Jurkat cell lysates had a concentration of $2\text{-}2.4 \times 10^7$ cells/ml. Aerosol deposition parameters were: biofluid flow rate, 800 nl/s; slide deck velocity, 2.54 cm/s; distance of nozzle to the slide, 2.54 cm and nozzle orifice diameter: 0.09 inches.

Kinase Assay Chips

The IQ™ assay solutions were prepared as suggested by the manufacturer except that 40% glycerol was mixed into the reaction buffer. Fluorescently tagged substrate was arrayed with or without 50 µM inhibitor (TYADFIASGRTGRRNAI-MH2, Upstate Biotechnology, Lake Placid, NY), ATP and PKA were then sprayed to activate the kinase reaction. After 2-3 hours incubation, quencher solution was sprayed and the fluorescent signal change was recorded. When a no PKA subarray was used as a control, PKA was arrayed together with substrate and inhibitor in the remainder of the subarrays, and the reactions were then activated by spraying ATP only.

Acknowledgements. We thank Dr. Scott Diamond, the inventor of the aerosol deposition technology for delivering biofluid on microarrays, and for both technical assistance and invaluable scientific discussion.

References

1. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S, Houfek, T, et al. (2000). Analysis of yeast protein kinases using protein chips. *Nature Genetics* **26**, 283-289.
2. Zhu, H., Klemic, J.F., Chang, S., Bertone, P., Casamayor, A., Klemic, K.G., Smith, D., Gerstein, M., Reed, M.A. and Snyder, M. (2001). Global analysis of protein activities using proteome chips. *Science* **293**, 2101-2105.
3. 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**, 270-274.
4. MacBeath, G. and Schreiber, S.L. (2000). Printing proteins as microarrays for high-throughput function determination. *Science* **289**, 1760-1763.
5. 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.
6. De Wildt, R.M.T., Mundy, C. R., Gorick, B.D. and Tomlinson, I.M. (2000). Antibody arrays for high-throughput screening of antibody-antigen interactions. *Nat. Biotechnol*. **18**, 989-994.
7. Nicholson, D.W. and Thornberry, N.A. (1997). Caspases: killer proteases. *Trends Biochem. Sci*. **22**, 299-306.
8. Lee, D., Long, S.A., Adams, J.L., Chan, G., Vaidya, K.S., Francis, T.A., Kikly, K., Winkler, J.D, Sung, C.M, Debouck, C. et al. (2000). Potent and selective nonpeptide inhibitors of caspases 3 and 7 inhibit apoptosis and maintain cell functionality. *J. Biol. Chem*. **275**, 16007-16014.
9. Webber, S.E., Tikhe, J., Worland, S.T., Fuhrman, S.A., Hendrickson, T.F., Matthews, D.A., Love, R.A., Patick, A.K., Meador, J.W., Ferre, R.A. et al. (1996). Design, synthesis, and evaluation of nonpeptidic inhibitors of human rhinovirus 3C protease. *J. Med. Chem*. **39**, 5072-5082.
10. Knezevic, V., Leethanakul, C., Bichsel, V.E., Worth, J.M., Prabhu, V.V., Gutkind, J.S., Liotta, L.A., Munson, P.J., Petricoin, E.F. 3rd, Krizman, D.B. et al. (2001). Proteomic profiling of the cancer microenvironment by antibody arrays. *Proteomics* **10**, 1271-1278.
11. Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P. et al. (1997). A combinatorial approach defines specificities of members of the caspase family and Granzyme B. *J. Biol. Chem*. **272**, 17907-17911.
12. Amstad, P.A., Yu, G., Johnson, G.L., Lee, B.W., Dhawan, S. and Phelps, D.J. (2001). Detection of caspase activation in situ by fluorochrome-labeled caspase inhibitors. *Biotechniques* **3**, 608-610.
13. Talanian, R.V., Quinlan, C., Trautz, S., Hackett, M.C., Mankovich, J.A., Banach, D., Ghayur, T., Brady, K.D., Wong, W.W. et al. (1997). Substrate specificities of caspase family proteases. *J. Biol. Chem*. **272**, 9677-9682.
14. Garcia-Calvo, M., Peterson, E.P., Leiting, B., Ruel, R., Nicholson, D.W. and Thornberry, N.A. (1998). Inhibition of human caspases by peptide-based and macromolecule inhibitors. *J. Biol. Chem*. **273**, 32608-32613.
15. Mitchell, P. (2002). A prospective on protein microarrays. *Nature Biotech*. **20**, 225-229.
16. Morita, T., Kato, H., Iwanaga, S., Takada, K. and Kimura, T. (1977). New fluorogenic substrates for α-thrombin, factor Xa, kallikreins, and urokinase. *J. Biochem*. **82**, 1495-1498.

17. Backes, B.J., Harris, J.L., Leonetti, F., Craik, C.S. and Ellman, J.A. (2000). Synthesis of positional-scanning libraries of fluorogenic peptide substrates to define the extended substrate specificity of plasmin and thrombin. *Nat. Biotechnol*. **18**, 187-193.
18. Butenas, S., Drungilaite, V. and Mann, K.G. (1995). Fluorogenic substrates for activated protein C: substrate structure-efficiency correlation. *Anal. Biochem*. **225**, 231-241.
19. Butenas, S., Ribarik, N. and Mann, K.G. (1993). Synthetic substrates for human factor VIIa and factor VIIa-tissue factor. *Biochem*. **32**, 6531-6538.
20. Harris, J.L., Backes, B.J., Leonetti, F., Mahrus, S., Ellman, J.A, Craik. C.S. (2000). Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. *Proc. Natl. Acad. Sci. U S A*. **97**, 7754-7759.
21. Toth, M.V. and Marshall, G.R. (1990). A simple, continuous fluorometric assay for HIV protease. *Intl. J. Pept. Protein Res*. **36**, 544-50.
22. Nishikata, M., Suzuki, K., Yoshimura, Y., Deyama, Y. and Matsumoto, A. (1999). A phosphotyrosine-containing quenched fluorogenic peptide as a novel substrate for protein tyrosine phosphatases. *Biochem. J.* **343**, 385-391.