A Homogenous Small Molecule Microarray for Enzymatic Assays

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ABSTRACT

Small molecule microarray as an emerging technology promises to play a pivotal role in chemical genetics. However, in spite of the fast development of this technology special requirements, such as the immobilization of small molecules on the chip surface limit the utilization of microarray. We have developed a unique homogenous microarray system that can be used to array any small chemical compounds, in addition of 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 following biochemical reactions are performed in a traditional solution-phase environment. Fluorescence based reaction signals were then scanned and analyzed with standard chip scanner and microarray analyzing software. In this study, we demonstrated that this chip 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 have identified potential "hits" after screening chips printed with small chemical compounds against caspases 1 and 3.

Keywords: small molecule microarray, peptide array, enzymatic assay, aerosol deposition, ultra high-throughput screening

1 THE HMM PLATFORM AND APPLICATIONS

1.1 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 (Fig. 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 (Fig. 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 (Fig. 1C).

1.2 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 2A 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 (Fig. 2B). The HMM system can also be adapted for other commercial available homogenous assays, such as the Kinase reaction illustrated in Figure 2C. 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 (Fig. 2C), and the fluorescence signal will be quenched upon the addition of a phosphate during kinase reaction (Fig. 2D). 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. 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.

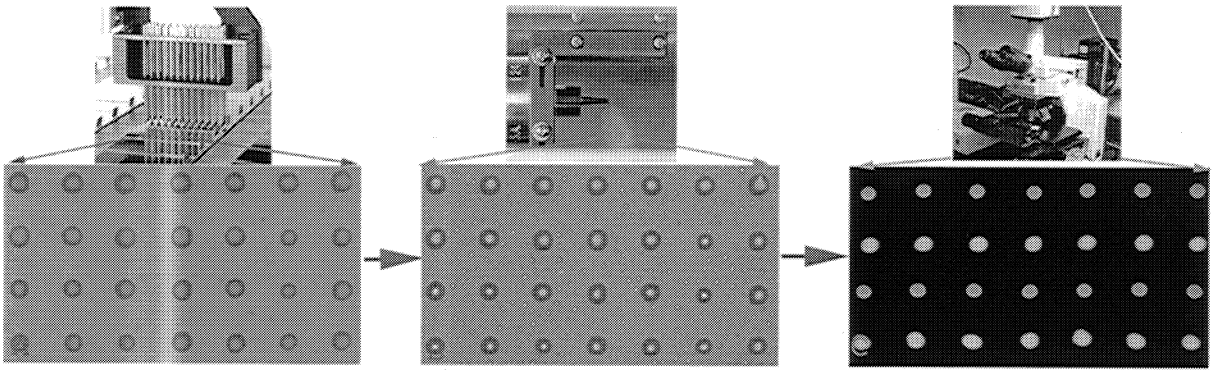

Fig. 1: HMM system. (A) Peptides, proteins or small molecules are mixed with reaction cocktail and then arrayed onto glass slides as individual reaction centers. (B) The chip is then activated by a fine aerosol mist of biological sample; the mist droplets fuse with each array dot without causing cross-contamination between reaction centers. (C) Fluorescent signals were detected with imaging instruments such as a fluorescence microscope, and the data was analyzed with microarray software.

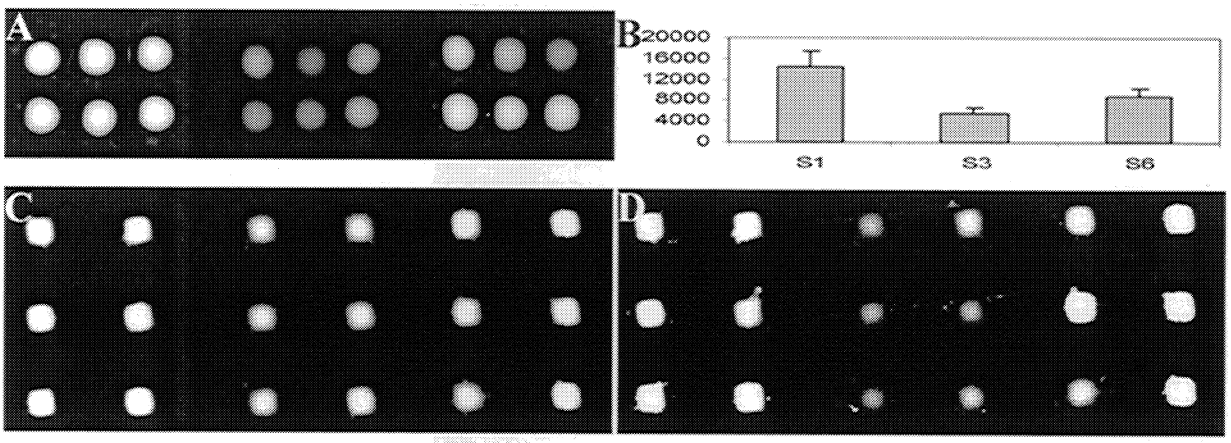

Fig. 2: Single enzymatic detection. Substrates for caspases 1, 3 and 6 were arrayed and activated by caspase 1 (A). The cross activities between caspase 1 with substrates of caspase 3 and 6 were well reflected in 384-well reaction, showed in (B). The fluorescently tagged PKA peptide substrate from the IQ™ assay kit was arrayed (C). The left subarray has substrate, inhibitor (50 mM) 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.

1.3 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 (Fig. 3). 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.

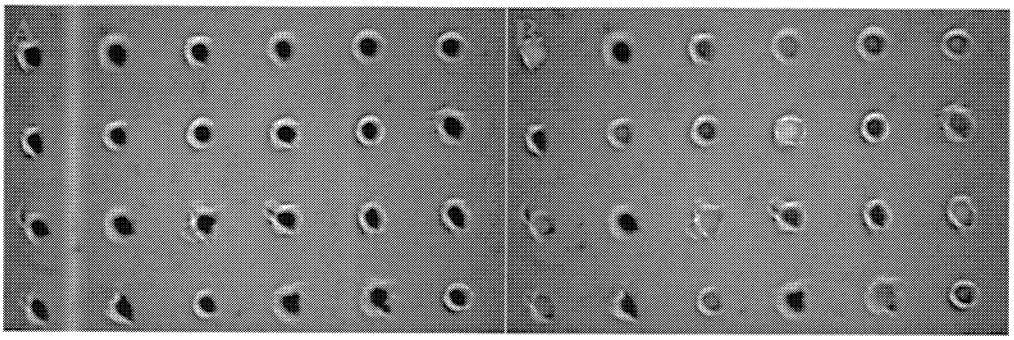

Fig. 3: Demonstration of Multiplex detections. Thrombin substrate with a blue fluorescent tag and chymotrypsin substrates with a red or green fluorescence tags were arrayed on the chip (A) and activated with thrombin and chymotrypsin simultaneously (B).

1.4 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 (Fig. 4). 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 used as positive controls to show inhibited activity (Fig. 4A and 4C). 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' (Fig. 4A and 4C). In this study, we found two potential inhibitors for caspase 3 (Fig. 4A and 4B) and one for Caspase 1 (Fig. 4C and 4D). We then repeated the same reactions in a 384-well format including the known inhibitor identified inhibitors and a randomly selected compound (C08). This experiment confirmed our microarray findings with caspase 3 (Fig. 4E) and caspase 1 (Fig. 4F).

1.5 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, 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. 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 (Fig. 5A) and after (Fig. 5B) 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 (Fig. 5C). This assay indicated that the HMM chip is sensitive enough to develop a protein expression profile. Such a chip will have a significant impact on basic apoptosis research, caspase drug development and drug-drug interaction studies.

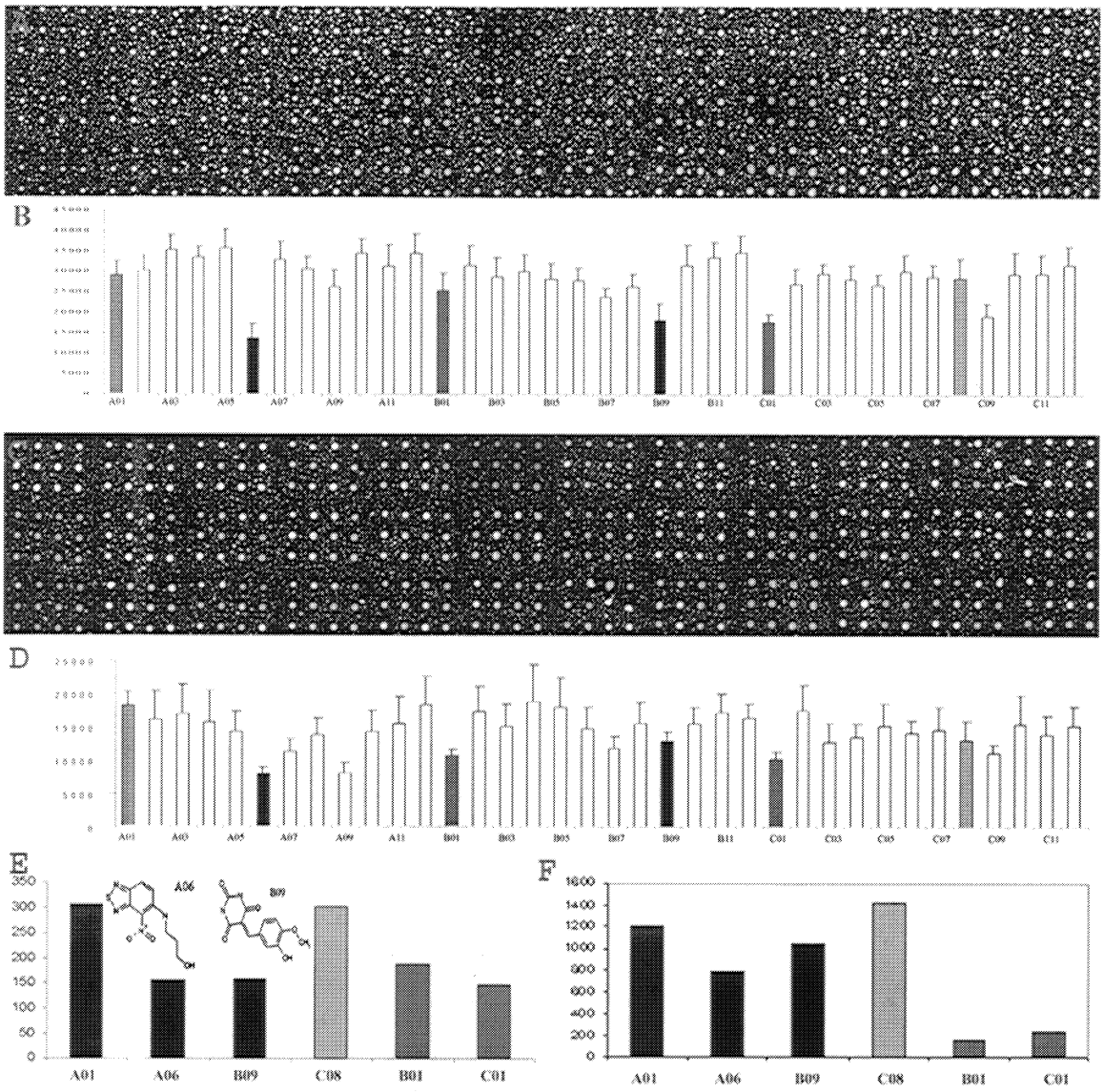

Fig. 4: An ultra high-throughput screening assay with the HMM system. A library of small chemical compounds were arrayed on a chip and screened with caspase 3 (A and B) and caspase 1 (C and D) respectively. Subarray A01 (Blue bar) is a negative control and subarrays B01 and C01 (red bars) are positive 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).

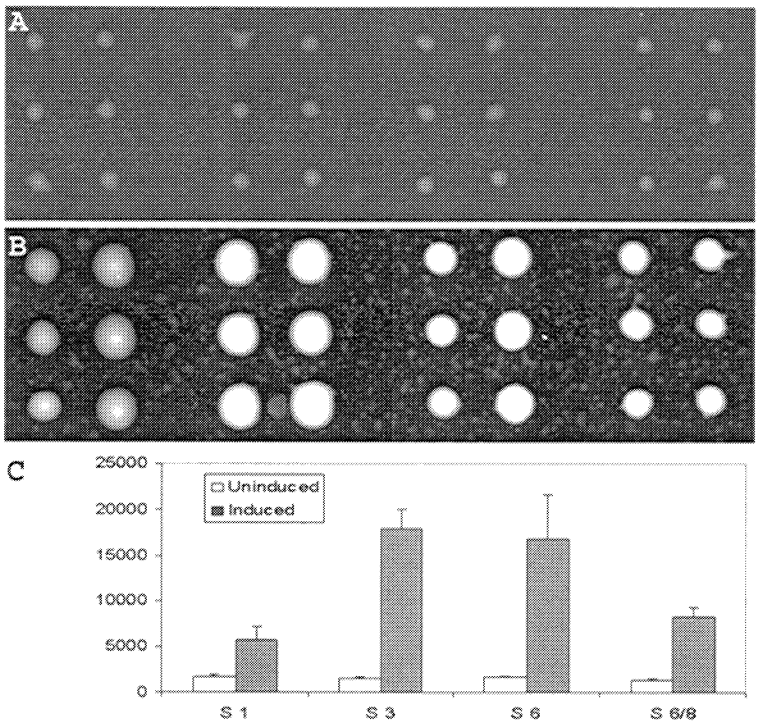

Fig. 5: Monitoring apoptosis pathways with whole cell lysates. Substrates of caspases 1, 3, 6 and 6/8 were arrayed and activated with Jurkat cell lysate before (A) and after (B) camptothecin induction. Campothecin induction increased caspase activities 3-9 fold (C).