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Nanodroplet profiling of enzymatic activities in a microarray

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Abstract—We describe a generic method for the large-scale functional characterization of enzymes in a microarray. Poly-L-lysine and amine reactive slides were coated with fluorogenic substrates sensitive to proteases and phosphatases. Patterning enzymes on the slides by robotic printing produced spatially addressable, segregated droplets that were simultaneously exposed to the on-chip sensors. Multiple enzymes were profiled using this system that provided fluorescence readouts across temporal and stoichiometric dimensions concurrently on a single microarray substrate. This integrated microarray platform is applicable not only for the functional annotation of proteins, but also for the rapid agonist and antagonist discovery and in performing on-chip kinetics.

The expanding repertoire of efficacious microarray technologies is in many ways revolutionizing proteomic research. Such wide-ranging impact is attributed to a variety of features that make array-based methodologies particularly attractive—the most significant of which being the ability to screen thousands of varied interactions simultaneously in a miniaturized scale.[1] This has given rise to a powerful analytic tool for dissecting the complex cellular circuitry, developing minute antibody-based platforms for diagnostics and the discovery of lead compounds as novel therapeutics. Our present work serves to add rapid enzyme characterization to the ever expanding list of microarray applications.[2]

Enzymes are biocatalysts intimately involved with vital cellular processes and metabolic exchanges. These proteins are suitable candidates for the directed perturbation of cellular functions and serve as valuable therapeutic targets. Minor imbalances in enzymatic expression are well documented to cause debilitating diseases. Amongst different classes of enzymes, proteases are known to participate in numerous physiological processes such as cell growth and differentiation, cell–cell communication and cell death. Phosphatases and kinases are responsible for the phosphorylation/dephosphorylation of biomolecules, intricate control over which forms the basis of signal transduction and cellular communication. Consequently, much effort has been put towards for a better understanding of the activity, biochemistry and cellular pathways controlled by enzymes, as well as in seeking molecules to modulate their activities.

Most existing microarray methods rely on the detection of strong, non-covalent interactions between potential 'hit' molecules and proteins immobilized on the array.[3] This has however limited microarray applications to those involving simple ligand binding exchanges, precluding key classes of proteins, such as enzymes, from being effectively characterized on arrays. Several groups have recently addressed this by developing various microarray-based platforms for the high-throughput screening of different classes of enzymes.[4–8]

Of the different methods used to detect enzymes, Salisbury et al. and our group independently used coumarin-containing enzyme substrate derivatives immobilized in a microarray to screen against different hydrolytic enzymes.[5,6] Selective enzymatic cleavage of the fluorogenic coumarins produced distinct fluorescence patterns, thus revealing the corresponding substrate-dependent enzyme profiles. In a related development, Gosalia and Diamond screened small molecules printed in glycerol droplets on a microarray for their ability to inhibit caspase activity.[8] The caspases were applied followed by a fluorogenic coumarin-containing caspase substrate, via aerosol. Though this facilitated miniaturized profiling of enzyme activity for inhibitor screening, the necessary use of low-volatile

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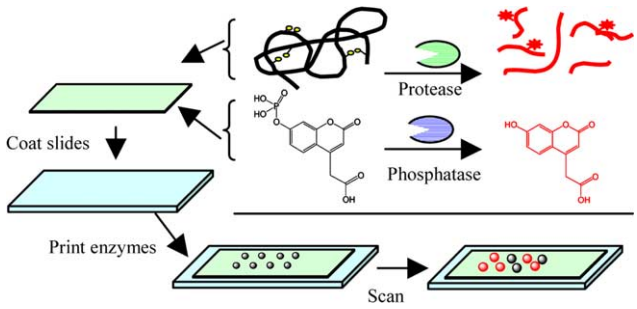
mediums such as glycerol and DMSO limits overall applicability in scenarios requiring predominantly aqueous environments. Although the strategy is potentially applicable to the screening of multiple enzymes, it has thus far been demonstrated in a 'one slide-one enzyme' format. Herein, we report a new microarray-based platform for potential high-throughput profiling of enzymes (Scheme 1). We show that, by creating nanodroplets on slides that are precoated with fluorogenic substrates, the activity profiles of many enzymes could be readily obtained simultaneously in a truly 'one slide-many enzyme' format.

Our approach involved first coating slide surfaces homogeneously with two fluorogenic sensors that target phosphatases and proteases. We had previously developed a repertoire of coumarin-based sensors for phosphatases, epoxide hydrolases and other protease subclasses.[6,9] Phosphate-modified coumarin was used herein as a representative of this class of probes. Removal of the phosphate group from the coumarin, as a result of phosphatase-catalyzed cleavage reaction, restores the fluorescence of coumarin (Scheme 1; bottom reaction). The second fluorogenic substrate was a commercially available EnzChek Protease Assay™ (Molecular Probes, OR, USA) that employs an intramolecularly quenched bodipy–casein conjugate. The probes' fluorescence is recovered upon proteolytic cleavage (Scheme 1; top reaction) by an active protease. Separate methods were used to immobilize each fluorogenic substrate: briefly, (1) poly-L-lysine slides were reacted with a 250 μM solution of ACC phosphate buffered in PBS (pH 7.4). The slides were rinsed with water to remove unassociated probe and stored at 4 °C in the dark until ready for use; (2) 3 μL of an original working bodipy–casein stock was diluted into 25 μL PBS (pH 7.4) and applied to a *N*-hydroxysuccinimide derivatized slide, using the coverslip method as previously described.[10] After a 2 h incubation, the slide was quenched with PBS containing a 0.5 M glycine solution. The resulting casein-coated slide was stored as described earlier.[10] Both types of slides were stable for a month without an appreciable loss in activity, but were typically used within a week of preparation. A set of 39 proteins were prepared fresh from solid stocks (Sigma–Aldrich, USA) desalted by running through NAP-5 columns (Amersham Biosciences, UK), and titrated using the Bradford protein assay to within 0.3–3 mg/ml in PBS (pH 7.4). Enzymes were kept on ice until printing on an ESI SMA™ arrayer (Ontario, Canada) at a pitch of 350 μM. Slides were incubated in a humid chamber with saturation above ~85% to minimize evaporation of the droplets followed by analysis on an ArrayWoRx™ microarray scanner (Applied Precision, USA) equipped with the relevant filters for coumarin ($\lambda_{ex/em}$: 360/457 nm) and bodipy ($\lambda_{ex/em}$: 490/528 nm). Microtitre plate verification was carried out using standard protocols on a SpectraMAX™ Gemini XS fluorescence plate reader (Molecular Devices, USA).

We tested the strategy by first spotting serially diluted trypsin (concentrations spanning from 0.3 mg/ml to 0.3 μg/ml) on bodipy–casein coated slides (Fig. 1). The spot intensities obtained were averaged, normalized against background and plotted graphically. As little as 10 μg/ml of trypsin was detected using this method and saturation was achieved beyond 100 μg/μL. Notably, the same grids printed on uncoated regions of the same slide were expectedly blank (data not shown).

We next screened 37 enzymes (5 phosphatases, 4 lipases, 15 proteases and the remaining 12 from diverse classes), together with 2 non-enzymes (Table 1) for proteolytic activity on the casein-coated slide. For an easy comparison, each of all 39 proteins were prepared in the same buffer (e.g., PBS), then subsequently spotted onto the microarray to ensure all enzymatic reactions were



Scheme 1. A strategy for rapid screening of enzymes using microarrays. The substrates, bodipy–casein conjugate (top) and phosphate-modified coumarin (bottom), were used to coat glass slides, and subsequently screen against proteases and phosphatases, respectively.

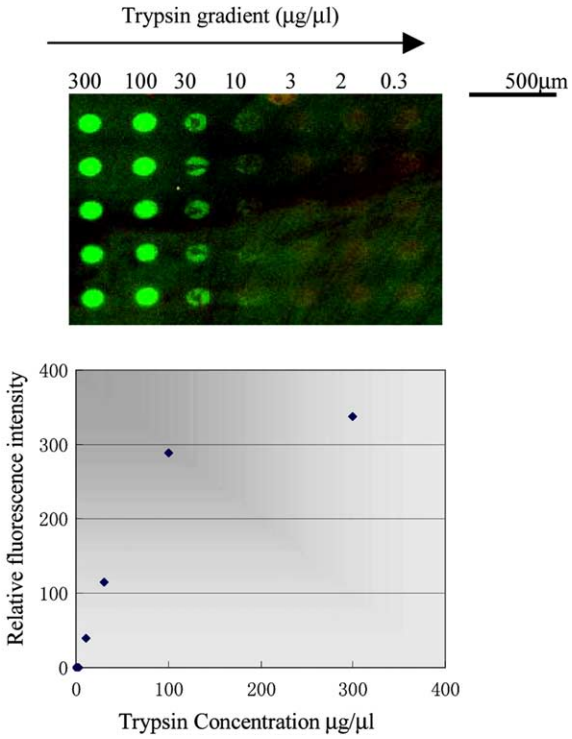


Figure 1. A 3-fold dilution series of trypsin printed on bodipy–casein coated slides scanned after 1 h of incubation (top). The enzyme was spotted in quintuplicate. The intensity profiles are represented by a graphical plot (bottom).

Table 1. A set of 39 proteins printed on bodipy–casein coated slides

1 Lysozyme	**14** α-chymotrypsin	**27** Amylose
2 Acid Phosphatase *1*	**15** β-chymotrypsin	**28** Concannavalin A (control)
3 Acid Phosphatase *2*	**16** Papain	**29** Alcoholdehydrogenase *2*
4 Alkaline Phosphatase *1 (AP1)*	**17** Pepsin	**30** Carbonic anhydrase
5 Alkaline Phosphatase *2(AP2)*	**18** Proteinase K	**31** PEP Carboxylase
6 Alkaline Phosphatase *3 (AP3)*	**19** Achromopeptidase	**32** Pyrophosphatase
7 Lipase *1*	**20** Penicillin amidase	**33** Trypsin
8 Lipase *2*	**21** Esterase	**34** Protease
9 Lipase *3*	**22** Epoxide hydrolase 1	**35** Thermolysin
10 Lipase *4*	**23** Acetylcholine esterase	**36** 3CL Protease
11 Trypsin inhibitor (control)	**24** Epoxide hydrolase 2	**37** Actinase E
12 Bromelain	**25** Alcoholdehydrogenase *1*	**38** Subtilisin
13 Chymopapain	**26** Urease	**39** Ficin

*Coloured typeset represents intensities after a four hour incubation: Highly active - **Red** (> 500 RFU), Moderately active - **Orange** (200-499 RFU), Weakly active - **Green** (50-150 RFU) and Inactive - **Blue** (< 50 RFU).

carried out under identical conditions. In addition, solution-phased microplate experiments were performed concurrently, and the results were normalized and presented in Figure 2 (top: microplate; bottom: microarray): notably most proteins registered comparable fluorescence profiles in both microarray- and microplate-based formats, indicating the successful implementation of our enzyme profiling strategy in a microarray. A few proteins appeared to show different fluorescence profiles. A closer examination revealed that the discrepancy was largely due to the intensity difference in the fluorescence readouts generated under the two assay formats (i.e., microplate vs microarray). Experiments are currently underway to address this in greater detail. From the results, three proteases, actinase E, subtilisin and ficin gave the strongest fluorescent intensities in both the microarray and the microtitre plate experiments. On the other hand, cysteine proteases such as 3CL protease, bromelain, papain and chymopapain showed weak activity, presumably as a result of the standard buffering conditions used throughout (without

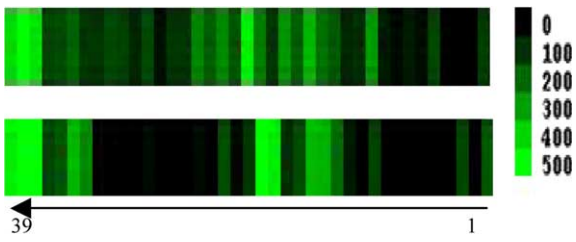


Figure 2. Profiles obtained using the 39 proteins in microtitre plate (top) and on microarray (bottom). Normalized values for each protein were entered and visualized in TreeView (http://rana.lbl.gov/EisenSoftware.htm). Intensities are represented by a colour bar, bright green representing the strongest values (scale inset).

dithiothreitol), which was perhaps not optimum. Pepsin, an aspartic protease also showing weak enzymatic activity in the microarray, was known to possess a maximum activity under acidic conditions. We wish to highlight that, while we chose to perform our current studies under uniform reaction conditions, one main advantage of our method is that, if desired, individual enzymes may be easily screened under different enzymatic conditions (e.g., buffer composition, salt, pH, additives, etc.) by simply reconstituting them with different buffers before spotting. Also intriguing from our results was the observation of a small number of non-protease proteins showing weak fluorescence profiles in both microarray and microplate experiments, indicating that these false-positive signals may have arisen from trace amounts of protease contaminations present in the commercial proteins.

Furthermore, by scanning the printed slides at different time intervals, we were able to map kinetic profiles of each of the proteins tested (Fig. 3a). The resultant profiles of eight representative proteins are plotted in Figure 3b. Actinase E gave the highest end-point intensity of 970 relative fluorescence units (RFU); its initial velocity was determined at 167 RFU/h. Subtilisin had however the highest initial velocity of 369 RFU/h with a relatively lower end-point value of 864 RFU. It was observed that for almost all the proteins, there was no increase in fluorescence signal beyond 4 h, indicating the reaction was complete.

Next we screened three different phosphatases on the coumarin-coated slides both in the presence and absence of a known inhibitor, sodium orthovanadate (Fig. 4). Control experiments carried out by spotting non-phosphatase proteins on the same slide did not generate any noticeable fluorescent spots (data not shown). The results shown in Figure 4 were independently validated using microplate experiments (data not shown). A 100 μM concentration of the inhibitor was found to completely inhibit all three phosphatases.

Central to our strategy is the application of enzymes onto the reactive array surface by robotic spotting. This facilitates the accurate and uniform deposition of suitably buffered enzymes, in volumes of approximately 1 pl. Essentially the procedure produced segregated droplets, each an independent microreactor. This allows a plethora of parameters to be varied, such as pH, buffers, ionic strength and presence of additives to be analyzed for individual or many enzymes simultaneously. Similarly, huge repertoires of inhibitors or promoters of enzymatic activity may be screened concurrently on a single microarray, allowing immediate verification of potentially valuable candidates. Though it is a concern in very large arrays that staggered protein deposition may attenuate exposure time, shortened printing duration may be easily achieved by increasing the number of print heads by up to 48 pins loaded on a standard microarray spotter, potentially allowing the arraying of a much larger number of proteins, in the order of minutes. Significantly, no-post-spotting processing was required, both solving the on-chip diffusion problem

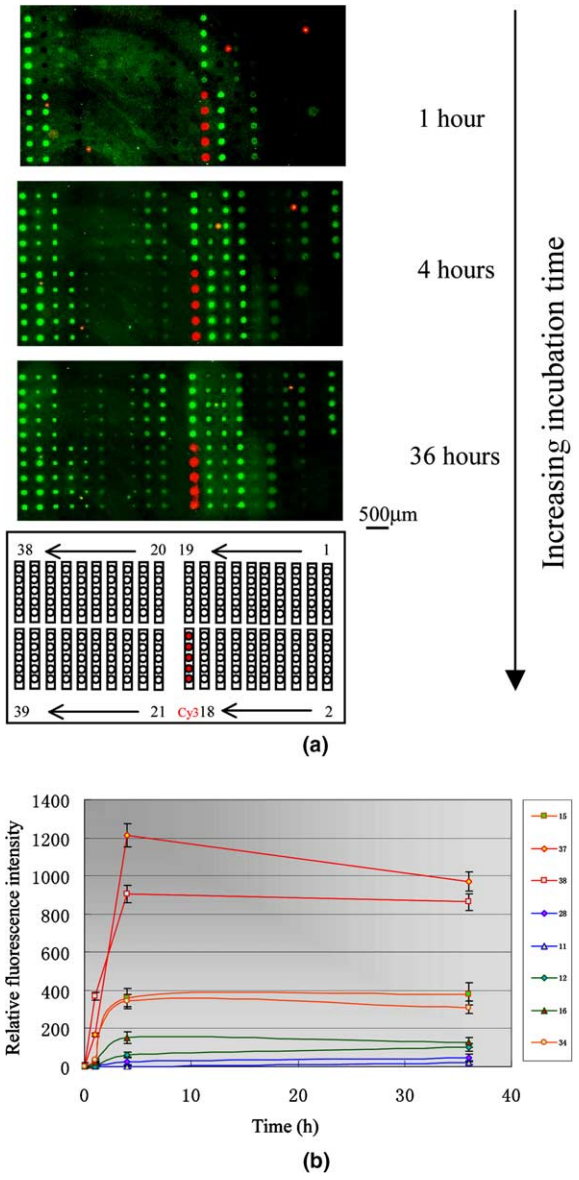


Figure 3. (a) Microarray images taken at different time points. Samples were printed in quintuplicate with the printing pattern shown. (b) The time-dependent activity profiles obtained of eight representative proteins (two from each category—strong, moderate, weak and inactive) was graphically presented.

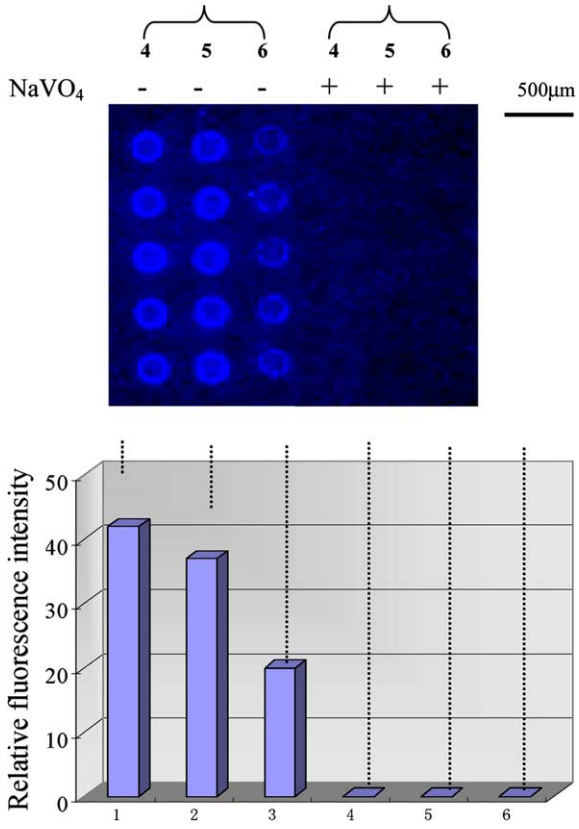


Figure 4. Phosphatase-sensitive slides screened against three representative alkaline phosphatases (**4**–**6**) in the presence and absence of an inhibitor, 100 μM sodium orthovanadate.

that arises when microarrays are washed, and minimizing the handling of slides. The strategy was also potentially cost-effective; the amount of the fluorogenic substrate required for a single reaction in a microplate experiment may be used for thousands of independent reactions on the array. For example, a mere 3 μL of the original working stock of bodipy–casein (sufficient for three microplate-based reactions) was sufficient to coat a 225 mm^2 slide surface on which we routinely performed up to 1260 separate assays—with space left over for a theoretical maximum of 5102 nanodroplets (at 350 μM spot spacing).

We had previously developed an alternative approach using activity-based probes with immobilized enzymes that can also cater to simultaneous profiling of many enzymes simultaneously on an array.[7] The present strategy is potentially more advantageous as highly variable conditions may be set up in each microreactor, maximizing throughput. There is also a wide range of untapped fluorogenic substrates that may be broadly exploited in a cost-effective manner on microarrays.[11] Furthermore, the use of enzymes in our strategy did not require their physical immobilization on the array and they were thus maintained in solution phase. This may provide a valuable feature to study on-chip kinetics of enzymatic reactions without having to consider the potential loss of enzyme activities or orientation upon immobilization.[3] Next generation microarray technology would have to move in line with the demands of post-genomic research, propelling the platform to new and exiting discoveries in the burgeoning proteomics arena. Our method reported here capitalizes on the numerous fundamental advantages provided by microarray-based technologies, and allows the potential high-throughput profiling of enzymes under a variety of different parameters. It may thus provide a useful tool for future research in proteomics and large-scale enzymology.

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