Nanodroplet Microarrays for High-Throughput Enzyme Screening

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Chapter 7

Nanodroplet Microarrays for High-Throughput Enzyme Screening

Kang L. D. Aw, Shao Q. Yao, and Mahesh Uttamchandani

Abstract

We describe here a method for the continuous assessment of enzymatic activity using microarrays. By uniformly coating fluorogenic substrates on slides, we generated surfaces capable of detecting enzymatic activity. The enzymes were deposited on the arrays in segregated droplets using standard microarrayers. Surfaces were developed for assessing the activities of both proteases and phosphatases, hence capitalizing on microarray technology to perform miniaturized high-throughput screens for these, as well as potentially any other, classes of enzyme. This offers an unprecedented ability for performing solution-phase enzymatic assays in *nanoliter* volumes on microarrays, in contrast to *microliter* volumes typically required in microplate-based assays, thereby reducing the amounts of reagent(s) required by anywhere from a hundred to a thousand-fold. This new approach thus provides a potentially more cost-effective, label-free enzyme screening technique. A single slide is able to accommodate several thousand assays, facilitating the assessment of both dose and time-dependent inhibition parameters in a single run.

Key words: Small molecule microarrays, High-throughput screening, Metalloproteases, Hydroxamate peptides, Enzyme assays, Chemical libraries, Inhibitor fingerprinting

1. Introduction

Enzymes are responsible for catalyzing all biological pathways, making them an indispensible group of proteins that support life in all its forms. Disruptions to enzymes and their functions can detrimentally affect cellular functions and metabolic exchanges, thereby causing a variety of diseases ranging from cancer and arthritis to Alzheimer's disease and cardiovascular disorders. A better handle over diseases may thus be achieved through a deeper understanding of the roles of enzymes and the processes they control. Platforms, such as the microarray, can facilitate the

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characterization and annotation of enzyme activity. We have developed a microarray workflow and methodology that enables not only the characterization of enzyme activity using microarrays, but also the screening of inhibitors in a high-throughput manner. This may be further applied to rapidly screen agonists or antagonists against target enzymes for the discovery of therapeutic leads.

Of the many different types of enzymes, proteases, kinases, and phosphatases are among the largest group of proteins and perhaps the most important (1). They have been involved in numerous physiological processes such as cell differentiation, signal transduction, host defense, and apoptosis (2). Minute alterations in expression and regulation of these enzymes can lead to debilitating effects. We have developed microarray surfaces that facilitate the screening of broad classes of enzymes, namely phosphatases and proteases. These arrays may be applied in two separate contexts. First is the ability to carry out functional annotation and discovery of enzymes (3). Second is the high-throughput screening of enzyme agonist or antagonists in an activity-dependent manner for lead discovery or protein fingerprinting (4). Both these approaches are illustrated here using metalloproteases as models. The method is, however, generic to many other classes of enzymes, as long as the corresponding fluorogenic enzyme substrate is available for application.

Our overall approach is illustrated in Fig. 1. Using this approach, one is able to carry out activity-based characterization as well as inhibitor screens of multiple enzymes simultaneously on a single glass slide. Key to our strategy is the application of enzymes by robotic spotting onto surfaces coated with fluorogenic substrates. This microarrayer dispenses subnanoliter volumes accurately and uniformly at predefined locations across the glass slide, thereby creating individual microreactors. This facilitates the variation of different parameters such as buffer conditions, pH, across a series of enzymes for simultaneous comparison and testing. A traditional microplate will require about 100 μL of flourogenic substrate (in this case, Bodipy Casein) for a hundred arrays of reaction wells, whereas just 6 μL of flourogenic substrate is sufficient for 6,148 separate microassays conducted on a slide surface surface of 880 mm^2 (250 μm spot diameter and a spot spacing of 500 μm), thereby enhancing throughput and reducing screening costs. Additionally, the use of enzymes in our approach requires no physical immobilization of the enzymes, thereby overcoming the usual limitation of microarrays where enzymes or inhibitors have to be immobilized or labeled resulting in a loss of activity or unfavorable orientations.

Several groups have also explored similar strategies (5). Salisbury et al. have used peptide derivatives containing coumarin as fluorogenic substrates to screen against different hydrolytic enzymes on microarrays (6). The selective hydrolysis of flourogenic

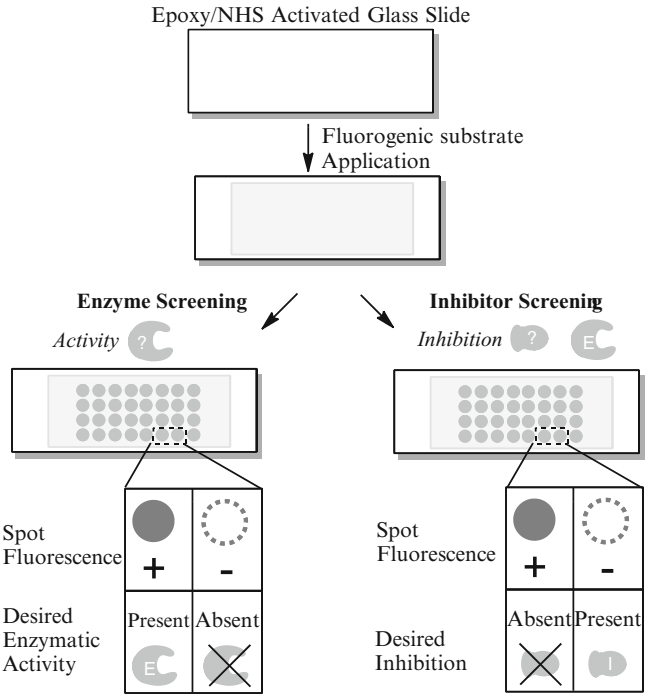


Fig. 1. A strategy for rapid screening of enzymes using microarrays. Fluorogenic substrates were first used to coat glass slides. These coated slides may then be applied either to screen proteins for annotation of their function, or for the discovery of inhibitors against target enzymes of interest.

coumarins through enzymatic activity produced patterns of fluorescence, thus revealing the enzyme's substrate preference. Gosalia and Diamond also printed small molecules in glycerol droplets on a microarray, followed by enzyme application via aerosol. This was applied for inhibitor screening of caspases and serine proteases (7, 8). These strategies only enable, however, one enzyme to be studied on any given slide. Our strategy, in comparison, offers the versatility for both enzymes and inhibitors to be analyzed simultaneously on the same slide. It thus provides an attractive solution for high-throughput enzyme screening.

2. Materials

2.1. Fabrication of Epoxy Slides

1. Sulfuric acid.
2. 30% hydrogen peroxide (Kanto Chemicals).
3. 1% 3-glycidopropyltrimethoxysilane (Sigma-aldrich, cat. No. 440167).
4. Acetic acid.
5. Ethanol.

6. MilliQ H_2O (18.2 Ω, 4 ppb).
7. Nitrogen gas.

2.2. Bodipy Casein Slides Derivatization

1. 1.0 mg/mL Bodipy Casein Working solution: 200 µL of 0.1 M sodium carbonate, pH 8.3 is added to the lyophilized substrate of EnzChek Protease Assay Kit (Invitrogen). Either Tr-X bodipy casein or bodipy casein may be used, as long as the scanner is equipped with the appropriate filter sets, 589/617 nm and 490/528 nm, respectively (see Note 1).
2. Quenching solution: 0.5 mM glycine solution in phosphate buffered solution (PBS) pH 7.4.

2.3. Printing Slides

1. 10–0.01 mg/mL of enzymes prepared in 50 mM Tris-HCl buffer (pH 8.0, 5% glycerol).

2.4. Synthesis of Small molecule Library on Solid Support

1. Rink amide-AM resin (GL Biochem).
2. Hydrochloric acid.
3. 20 proteinogenic Fmoc protected amino acids (GL Biochem).
4. Fmoc-Lys(Biotin)-OH (GL Biochem).
5. O-Benzotriazole- *N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU; GL Biochem).
6. O-(7-Azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) (GL Biochem).
7. N-Hydroxybenzotriazole (HOBt) (GL Biochem).
8. 2,4,6-Collidine (Sigma-Aldrich).
9. Piperidine (Acros Organics).
10. Trifluoroacetic acid (TFA) (Sigma-Aldrich).
11. Triisopropylsilane (TIS) (Sigma-Aldrich).
12. *N*,*N*-Diisipropylethylamine (DIEA) (Sigma).
13. Solvents: Acetone, Acetonitrile, Dichloromethane, Diethyl ether, Dimethylformamide (DMF), Methanol (Tedia).

2.5. Equipment

1. Clean Glass Slides (22 × 60 mm) (Fisher Scientific, Pittsburgh, PA).
2. Microscope Cover Slips (22 × 40 × 0.15 mm) (Matsunami Glass, Japan).
3. Metal slide racks and glass slide staining dish (Electron Microscopy Sciences).
4. Polypropylene slide staining dish and rack (Kartell).
5. Stealth Micro Spotting Pins (TeleChem International, Ca).
6. OmniGrid Microarrayer (Harvard Bioscience, Massachusetts).

7. GenePix 4000B Scanner (MDS Analytical Technologies, Ontario).
8. LC-MS workstation.

2.6. Software

1. OmniGrid Gridder Software (or equivalent).
2. Gene Pix Pro Microarray Image Analysis Software (or equivalent).
3. Microsoft Excel (or equivalent).

3. Methods

The strategy works by coating glass slides surfaces homogenously with commercially available fluorogenic sensors that target proteases. The substrate used employ intramolecularly quenched casein derivatives, which upon hydrolysis, releases a strong fluorescence readout. The fluorescence intensity is proportional to the activity of the protease, thus allowing activity-based measurements to be performed. Typical results obtained are showcased in Figs. 2 and 3.

3.1. Fabrication of Epoxy Slides

Glass slides are cleansed and soaked in piranha solution (70% sulfuric acid: 30% hydrogen peroxide) to remove any organic contaminants and oxidize the silane surface (see Note 2).

1. Use the polypropylene staining dish to contain the piranha solution. The slides may remain soaked in this solution till ready to use (see Note 3).
2. The slides are thereafter transferred to a stainless steel rack and washed copiously with distilled water and dried.
3. Prepare a 400 mL solution containing 1% 3-glycidopropyltrimethoxysilane in 95% ethanol containing 16 mM acetic acid (sufficient for one rack of 30 slides).
4. Premix the solutions in a glass jar staining jar for 10 min and add in the slide in the metal rack. Continuously stir the solution using a magnetic stirrer.
5. Incubate the slides for 1–2 h.
6. Remove the slides from the solution and rinse copiously with ethanol. Air-dry the slides to remove any residual ethanol.
7. Transfer the slide tray to a deep well dish and cure at 150°C for at least 2 h.
8. Subsequently, cool the slides to room temperature and rinse them with ethanol. Air-dry the slides or to speed up the drying

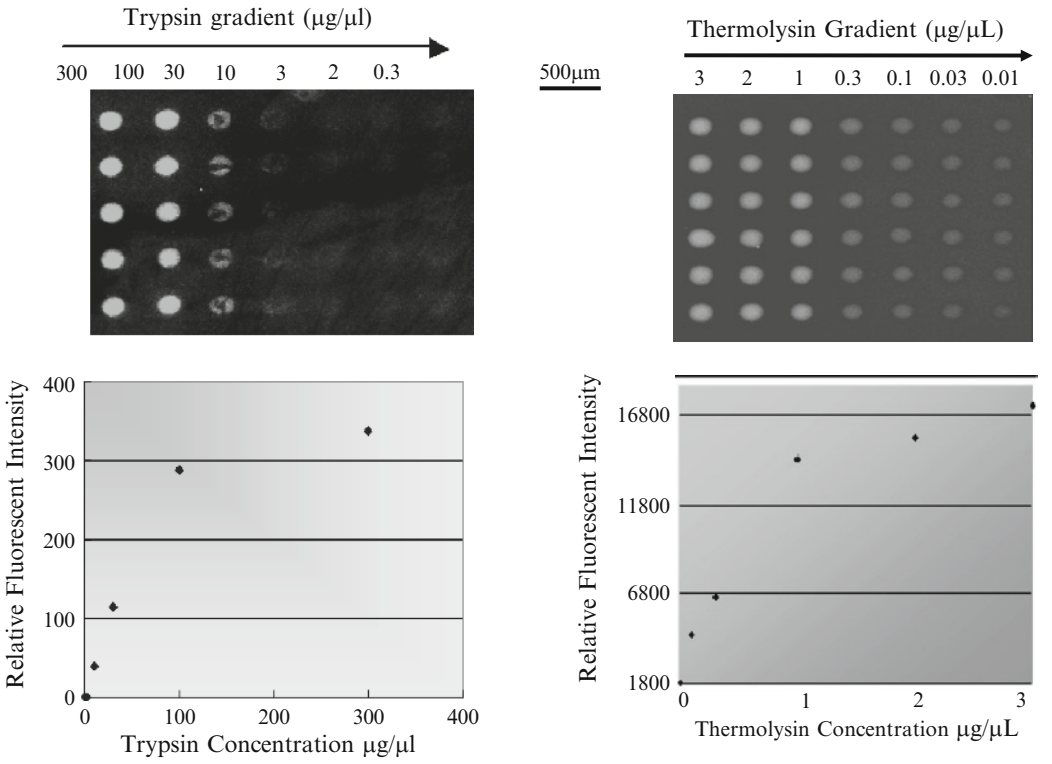


Fig. 2. Dilution series of trypsin and thermolysin respectively printed on bodipy casein coated slides. The intensity profiles are represented by a graphical plot (bottom). (Reprinted from ref 3 with permission from Elsevier).

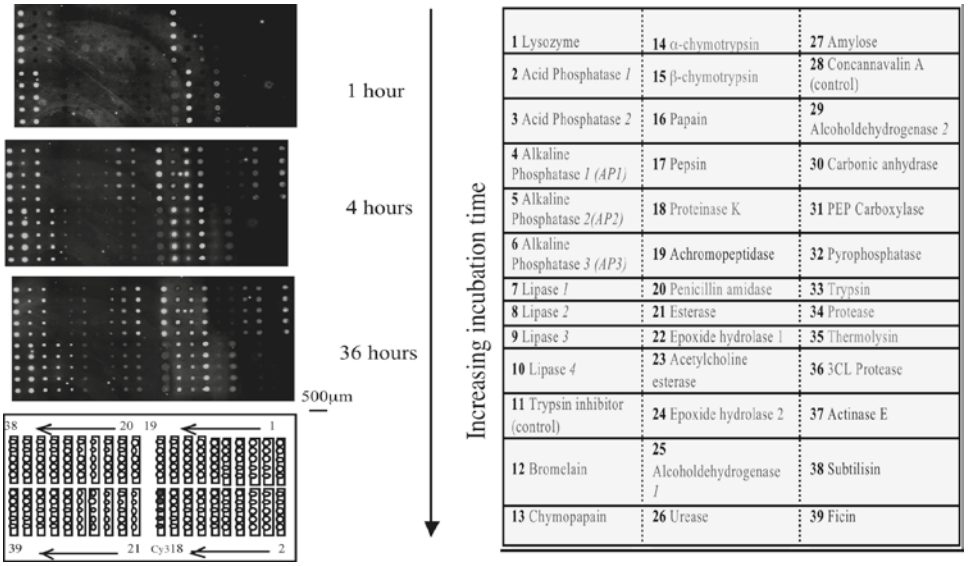


Fig. 3. Microarray images taken at different time points for a panel of enzymes on bodipy casein slides. It can be seen that positive measurements of proteolytic activities were obtained in a time-dependent manner. The controls or nonproteolytic enzymes did not display significant fluorescent readouts. (Reprinted from ref 3 with permission from Elsevier).

process, use centrifugation or a stream of dry nitrogen/compressed air to dry the slides.

9. Slides may be stored at room temperature until ready for use.

3.2. Preparing Bodipy Casein Slides

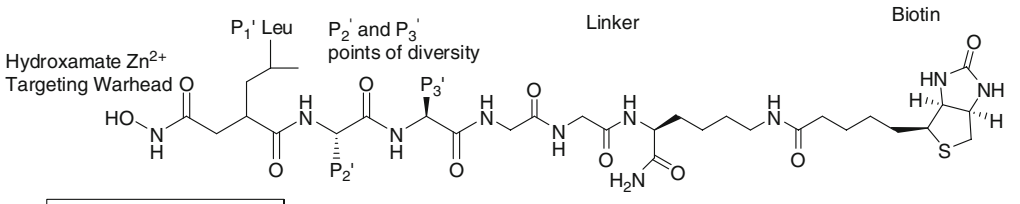
1. Dilute 6 μL of the original working stock solution of bodipy casein in 44 μL of PBS.
2. Apply reaction mix on epoxide-functionalized slides using cover slip method (see Note 4)
3. Incubate slides for 2 h in an enclosed light protected humid chamber to prevent drying up of the reaction mix.
4. After 2 h of incubation, quenched slides are quenched in 0.5 mM glycine in 1× PBS buffer.
5. Rinse in MilliQ water and dry. Store at 4°C, in the dark.

3.3. Printing Enzymes on Microarrays to Detect Enzymatic Activity

1. Prepare the enzymes at desired concentrations (usually between 10 and 0.01 mg/mL) in 50 mM Tris-HCl buffer (pH 8.0, 15% glycerol) (see Note 5).
2. The enzymes are then dispensed into 384 wells plates using multichannel pipettes.
3. Shake the 384 wells plate to mix and spin down. The plate is kept on ice until printing.
4. Design grids using the gridder software to plan the arrangement of the spots on the slide such that the enzymes are spotted at least twice on the slide. Blotting may be incorporated into the printing schedule to improve spot quality across the slide.
5. Rinse to rehydrate the coated slides and dry prior to loading the slides on the spotter. Ensure the TR-X casein coated surface is facing upward.
6. Spot the enzymes using an OmniGrid Microarrayer with a spot spacing of 500 μm using a SMP8B pin, creating spots of diameter of approximately 350 μm. (see Notes 6 and 7)
7. Slides are printed in a humid environment with saturation of 85% to minimize evaporation of the droplets and in the absence of light.

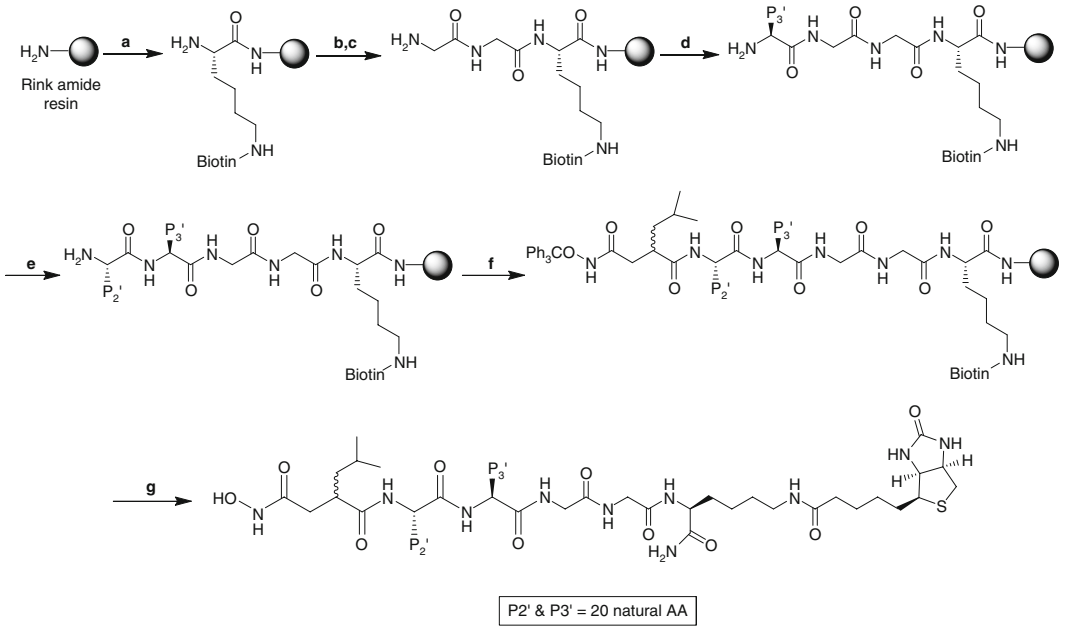
3.4. Design and Synthesis of Inhibitor Library

The design of the small molecule library is shown in Fig. 4 that targets metalloproteases. The synthetic scheme is provided in Scheme 1. The synthesis of the panel of 14 different trityl-protected hydroxamate warheads have been described previously (9). Here, the protocol is described for the synthesis of a 400 member library, using Fmoc solid phase peptide synthesis (10). The procedure may be modified as desired to create various points of



P_1'	P_2'	P_3'	Library Size
L	20 natural amino acids	20 natural amino acids	400 members
1 P_1' warhead			Total: 400 compounds

Fig. 4. Design of small molecule inhibitor library. A 400 member hydroxamate peptide library was designed to target metalloproteases.



Scheme 1. Synthesis scheme of small molecule library. (**a**) (1) Fmoc-Lys(Biotin)-OH, HOBT, HBTU, DIEA, DMF; (2) 20% piperidine/DMF; (**b**) (1) Fmoc-Gly-OH, HOBT, HBTU, DIEA; (2) 20% piperidine/DMF; (**c**) (1) Fmoc-Gly-OH, HOBT, HBTU, DIEA; (2) 20% piperidine/DMF; (**d**) (1) Fmoc-AA(P_3)-COOH, HOBT, HBTU, DIEA; (2) 20% piperidine/ DMF; (**e**) (1) Fmoc-AA(P_2)-COOH, HOBT, HBTU, DIEA; (2) 20% piperidine/DMF; (**f**) (1) 4, CPh_3ONH-Leu-COOH, HATU, 2,4,6-collidine; (2) 20% piperidine/DMF; (**g**) 95% TFA/5% TIS, 2 h.

diversity. It is also manageable for one person to handle this protocol with 400–500 MicroKans independently, which would consume a period of between 8 and 12 days.

1. Calculate the amount of rink amide resin required for library synthesis. The capacity for each MicroKan is 30 mg of resin. This would work out to a total of 12 g of resin for a

400 member library. The theoretical yield of each library member at this scale of synthesis would be 15 μmoles, when using rink amide resin with 0.5 mmol/g resin loading capacity. This quantity is more than sufficient for printing several thousand microarray slides.

2. Weigh 12 g of rink amide resin into a fritted funnel, with a screw cap. Add 35 ml of DMF, cap both ends of the funnel and shake for 2 h to allow the resin to swell. Drain the DMF using suction.
3. Repeat the wash step with 35 ml DMF for a further three times, for 15 min each time with shaking.
4. Deprotect the resin using 20% v/v piperidine in DMF for 1 h with shaking. Drain the piperidine solution using suction.
5. Wash the resin with DMF (35 ml) for three times, 15 min per wash with shaking.
6. Wash with DCM (35 ml) for a further three times for 15 min per wash with shaking.
7. Wash with DMF (35 ml) for a further three times for 15 min per wash with shaking.
8. Transfer the resin from the funnel into a 250 ml glass bottle. Rinse with 25 ml of DMF to ensure all resin is completely transferred. Repeat with another 10 ml of DMF.
9. Couple resin with Fmoc-Lys(biotin)-OH. Weigh out four equivalents (24 mmol) of Fmoc-Lys(biotin)-OH, HBTU, and HOBt in a separate bottle. Dissolve in 80 ml of DMF and add 8 equivalents of DIEA (48 mmol). Mix well, and allow for preactivation by leaving the solution to stand for 15 min.
10. Add the preactivated solution to the resin. Seal the bottle with parafilm and shake overnight (~12 h).
11. Filter the resin using suction through a 350 ml fritted funnel. Ensure that all resin is transferred using small volume rinses with DMF.
12. Wash the resin using 3× DMF, 3× DCM, and 3× DMF, as described in steps 5–7, using 100 ml of solvent per wash.
13. Isolate around ten beads, and perform the ninhydrin test to ensure that the coupling is successful and complete (see Note 8).
14. Transfer the resin to a clean bottle and deprotect Fmoc using 150 ml of 20% piperidine in DMF.
15. Wash as detailed in step 12.
16. Repeat steps 8–15 using Fmoc-Gly-OH in place of Fmoc-Lys(biotin)-OH to couple a glycine residue.
17. Repeat step 16 to add on another glycine residue.

18. Dry the resin under vacuum, using an oil pump for 6 h.
19. Distribute ~30 mg of resin in each of 400 MicroKans reactors. Include an IRORI radiofrequency tag to each reactor and ensure that the cap is fitted on tightly. Load four extra reactors for ninhydrin tests to monitor coupling efficiency.
20. Program the Accutag Synthesis Manager software for a 20 aa × 20 aa = 400-member library.
21. Scan the 400 reactors to encode each tag and sort into twenty 100 ml bottles. Reactors in each bottle will be coupled with the same amino acid.
22. To couple the P_3' residues, prepare preactivated solutions for each of the 20 Fmoc-protected proteinogenic amino acids, using the four equivalent molar excess of amino acid, HBTU, HOBt, and eight equivalent molar excess of DIEA. Prepare these solutions in 50 ml of DMF.
23. Add the twenty different amino acid preactivated solutions to the 20 bottles containing the respective MicroKans. Ensure that all bottles are appropriately labeled.
24. Shake bottles overnight (~12 h).
25. Drain the solutions in each bottle and wash twice (for 15 min) with 60 ml of DMF.
26. Pool all reactors into a 1 L bottle and rinse using 3× DMF, 3× DCM and 3× DMF, as described in steps 5–7, using 500 ml of solvent per wash.
27. Perform Fmoc deprotection using 400 ml of 20% piperidine in DMF.
28. Wash as described in step 26.
29. Repeat steps 21–28 to sort the MicroKans into 20 bottles couple the P_2' position.
30. For the final coupling of the P_1' position, consolidate all MicroKans into a clean 1 L bottle. Proceed with steps 22–28, except use four equivalents of a trityl-protected hydroxamate warhead together with HATU and 2,4,6-collidine (in a 1:1:1.9 ratio) in a 100 ml DMF volume for the coupling step.
31. Perform a final wash with 500 ml of methanol for three times, with shaking each time for 15 min.
32. Dry the resin under vacuum, using an oil pump for 6 h.
33. Prepare the cleavage solution comprising TFA and TIS in the ratio of 19:1. Sort the 400 reactors into individually identified 15 ml tubes. Dispense 1.5 ml of the cleavage solution to each tube, and shake for 3 h. TFA is corrosive and generates fumes, and so perform this step carefully with proper protection in a fumehood.

34. Organize five 96-well deep well plates such that the identities of the samples will be preserved upon transferring all the solutions from each of the 400 15 ml tubes to the plates. Carefully transfer solutions from tubes to plate.
35. Concentrate and remove TFA and TIS using a vacuum evaporator, at a temperature of 35°C at a spin force of 300×*g*, until about 0.1 ml of the solution remains in each well.
36. Add 1.5 ml of cold ether to each well to precipitate the small molecule. Seal the plates with adhesive film and place in a −20°C freezer overnight.
37. Spin down the precipitated products at 1,000×*g* for 30 min, and decant the ether.
38. Dissolve the products in 0.5 ml of DMF. This would give stock concentrations in the range of 1–10 mM. Plates may be sealed and stored for the long term at −80°C.
39. Perform analysis using LC-MS to determine quality and purity of desired products.

3.5. Testing Enzyme Inhibition Using Nanodroplet Microarrays

1. Prepare the enzymes to a 0.5 mg/mL solution in 50 mM Tris-HCl buffer (pH 8.0, 15% glycerol) and mixed with approximately 2 μM of inhibitor.
2. The enzymes and inhibitors are then pipetted into 384 wells plates using multichannel pipettes.
3. Shake the 384 wells plate to mix and spin down. The plate is kept on ice until printing.
4. Design grids using the gridder software to plan the arrangement of the spots on the slide such that the enzymes are spotted at least twice on the slide. Blotting may be incorporated into the printing schedule to improve reproducibility within the slide.
5. Load the required pins and slides in the spotter and make sure the TR-X casein coated side is facing upward.
6. Spot the enzymes using an OmniGrid Microarrayer with a spot spacing of 500 μm using an SMP8B pin, creating spots of diameter of approximately 350 μm. (see Notes 7 and 8).
7. Slides are printed in a humid environment with saturation of 85% to minimize the evaporation of the droplets and if possible in the dark.

3.6. Scanning of the Slides

1. Scan slides on a microarray scanner equipped with the relevant filters. Depending on the nature of the experiment, slides may be scanned periodically, or after a fixed duration of incubation.
2. An example of the typical results obtained is displayed in Figs. 2 and 3 (concentration and time dependent, respectively).

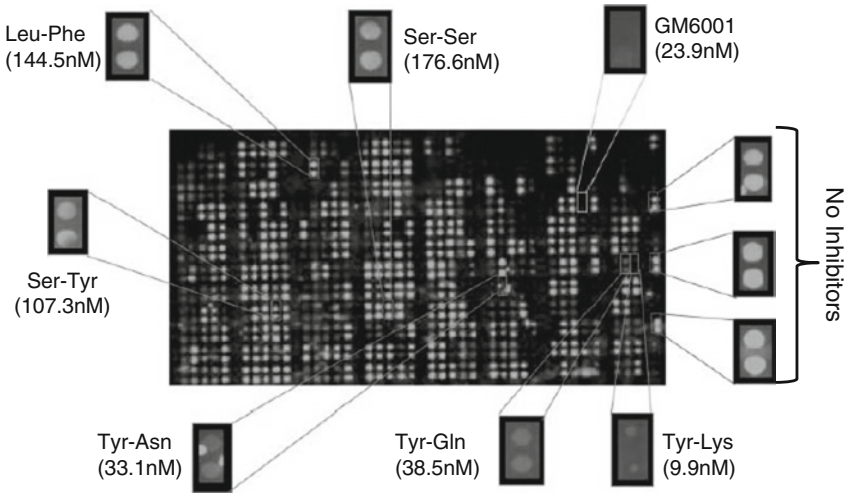


Fig. 5. Nanodroplet microarray results of the 400-member library screened against thermolysin. Samples were spotted in duplicate. Boxes magnify spots of selected inhibitors (labeled by their P_2'–P_3' sequence) with corresponding IC_{50} values in parenthesis. (Reprinted from ref 4 with permission from the Royal Society of Chemistry).

Inhibitor end-point screening results are shown in Fig. 5, with several inhibitors of varying potency displayed inset.

4. Notes

1. Reconstituted fluorogenic substrates can be stored for 2–4 weeks at 4°C. If longer storage is required, fluorogenic substrates should be kept at –20°C. Repeated freezing and thawing should also be avoided to preserve the viability of the substrate.
2. Care must be taken when handling slides to be used in microarray experiments to ensure that at all stages of derivatization, spotting and sample application no dust or dirt come into contact with the planar surfaces. Such particles may cause extraneous fluorescence or result in scratches that could affect the fluorescent readout when the slides are scanned. Ensure that all surfaces and slide racks are clean and rinse these surfaces with ethanol before placing in direct contact with the slides. Gloves, if used, should be of the powder-free variety to ensure that the slides remain uncontaminated even after handling.
3. Piranha solution is highly corrosive. Make sure that proper personal precautions are maintained when handling this solution. Add the hydrogen peroxide to the acid slowly, if not the solution can get very hot very quickly. Solution may be recycled, but is hygroscopic and can absorb moisture from the air

and become diluted over prolonged use. When this happens, prepare fresh solution. Most preparations may be used over 1–2 months.

4. For a 22×40 mm cover slip, a 50 μL reagent is adequate to allow for a uniform coating. Two methods can be used to apply the reagent to the slides. One can either apply the reaction mix to the glass slide followed by the cover slip over the reaction mix or one can apply the reaction mix to the cover slip and then followed by the glass slide onto the reaction mix. Although both methods work similarly and well enough, there are slight differences in the handling and maneuvering of the liquid droplet upon contact of both the slide and cover slip. Either method is fine as long as one is able to produce a uniform distribution of the reaction mix over the cover slip and glass slide without the introduction of air bubbles between the two surfaces.
5. It is preferable to prepare enzymes solution fresh, prior to use. Long-term storage or repeated thawing and freezing may degrade enzyme activity.
6. Spotting volume can be varied by using different pin head sizes. SMP3 stealth pins are very frequently used in microarray fabrication and generally produce spots approximately 100 μm in diameter. As a result, SMP3 pins are more economical in terms of reaction volume, and in addition allowing more nanodroplets to be printed on a single slide. Although this characteristic improves the cost-effectiveness of our proposed methodology, but we found out that when spotting viscous solution, such as enzymes prepared in glycerol, it is more advantageous to use larger pins, such as SMP8 (pins size we used in this proposed methodology), or even SMP15 stealth pins that produces spots in the range of 250–500 μm, respectively. Using SMP8 and SMP 15 pins, visualization and analysis of the spots on the scanner is generally easier. Furthermore, the use of SMP8 pins (pins size used in this methodology) displays a more desirable standard deviation in the reproducibility of the spots as compared to using SMP3, as shown in Fig. 6.
7. During the printing process, the pins are rinsed between samples using two cycles of wash (for 10 s) and sonication (for 20 s) in reservoirs containing 70% ethanol followed by drying under reduced pressure (for 20 s), causing a one minute interval in between samples printing. This interval can be easily changed using the gridder software of the machine, allowing the user to determine the interval required and thus carrying out time and concentration dependent slides printing simultaneously on a single glass slide. As such, the arrayer

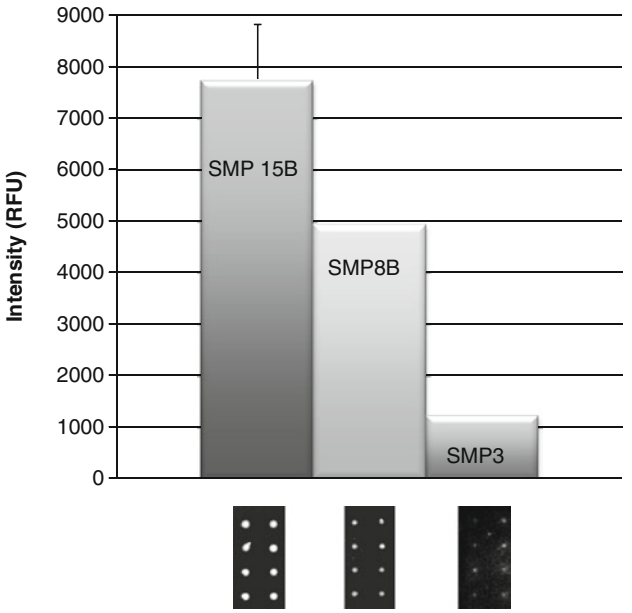


Fig. 6. Thermolysin spots at 1.0 mg/mL on a TR-X bodipy coated Slide using SMP15B, SMP8B, SMP3 pins, and the average plotted values of each corresponding spots. The error bars in the graphs correspond to the standard deviation across the group.

is able to print rows of samples with a fixed time interval in between. This allows the full kinetic read of the samples in a single slide scan.

8. The presence of a blue coloration on the resin and/or solution implies the presence of free amines, and hence indicating incomplete coupling. On the other hand, a straw yellow colour indicates no free amines, and complete coupling. Repeat coupling, if necessary, until a straw yellow colour is obtained. Alternatively, if coupling remains incomplete after several tries, capping may be performed using acetic anhydride.

Acknowledgments

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