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High Throughput Screening Using Enzyme Assay Microarrays

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Abstract

We report a new slide based microarray platform for assaying multiple enzyme activities using fluorogenic substrates. The method enables us to achieve the microfluidic requirements for rapid reaction assembly and compartmentalization. We can thus determine enzymatic activities in individually controlled reaction environments containing cofactors, inhibitors and activators. Fluorogenic substrates in glycerol were arrayed onto glass slides with reaction volumes < 5 nL and feature sizes of < 150 μm . Our method allowed rapid multiple sample deliveries onto the slide (< 3 nL/spot) with no cross contamination between array positions. It enabled us to detect the activation of the fibrinolytic and coagulation proteases namely, thrombin, plasmin, factor Xa, tPa and kallikrein in human plasma. Enzyme-substrate-inhibitor assays using ten caspases were also performed. With over 400 spots/cm², combinatorial substrate libraries with different proteases can now be rapidly profiled. An assay to detect the dose response of a thrombin inhibitor benzamidine was performed. The inhibitor was arrayed in replicates onto selected positions on the chip. After sequential subnanoliter delivery of the reaction components, the result from the array was analyzed. The expected dose response from benzamidine was seen. A CV of 5.26% was achieved for 232 positions on the array not spiked with the inhibitor. Thus, with potentially several thousand compounds per slide, using rapid sub-nanoliter delivery of components and standard equipment, the true potential of the method is in the field of high throughput screening.

Keywords

microarray, fluorogenic, enzyme, high throughput screening

Comments

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HIGH THROUGHPUT SCREENING USING ENZYME ASSAY MICROARRAYS

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Abstract-We report a new slide based microarray platform for assaying multiple enzyme activities using fluorogenic substrates. The method enables us to achieve the microfluidic requirements for rapid reaction assembly and compartmentalization. We can thus determine enzymatic activities in individually controlled reaction environments containing cofactors, inhibitors and activators. Fluorogenic substrates in glycerol were arrayed onto glass slides with reaction volumes < 5 nL and feature sizes of <150µm. Our method allowed rapid multiple sample deliveries onto the slide (<3nL/spot) with no cross contamination between array positions. It enabled us to detect the activation of the fibrinolytic and coagulation proteases namely, thrombin, plasmin, factor Xa, tPa and kallikrein in human plasma. Enzyme – substrate – inhibitor assays using ten caspases were also performed. With over 400 spots/cm², combinatorial substrate libraries with different proteases can now be rapidly profiled. An assay to detect the dose response of a thrombin inhibitor benzamidine was performed. The inhibitor was arrayed in replicates onto selected positions on the chip. After sequential subnanoliter delivery of the reaction components, the result from the array was analyzed. The expected dose response from benzamidine was seen. A CV of 5.26% was achieved for 232 positions on the array not spiked with the inhibitor. Thus, with potentially several thousand compounds per slide, using rapid sub – nanoliter delivery of components and standard equipment, the true potential of the method is in the field of high throughput screening.

Keywords - microarray, fluorogenic, enzyme, high throughput screening

I. INTRODUCTION

Gene expression analysis is unable to measure post translational modifications or protein – protein interactions. Recent studies have given evidence that there may be no strict correlation between the mRNA expressed and proteins levels in a cell. [1,2] With vast amounts of genomic data being generated, a high throughput, highly sensitive reliable method to determine the encoded genes and proteins has become necessary.

Protein chip technology has the characteristics of high throughput and high sensitivity. These chips utilize proteins, protein fragments, peptides and antibodies immobilized onto surfaces such as chemically modified glass slides [3], agarose films[4], photolithographed glass[5], silica fibers[6], polyacrylamide gel pads[7,8] and microwells[9]. The immobilized proteins, peptides or antibodies are then treated with samples of interest using labeled probes similar to DNA chips. The probes are then detected using methods best suited for the detection of the given label. This technique has the same functionality as the DNA chip. Protein chips can

thus be used to detect 'biochemical activities, protein – protein, protein – DNA, protein – RNA and protein – ligand interactions[9].'

MacBeath and Schreiber tested the feasibility of using glass as a solid phase surface for protein chips. They arrayed proteins in 40% glycerol and phosphate buffered saline to modified glass slides and tested its feasibility for protein – protein interactions, kinase substrate reactions and protein – small molecule interaction[3]. Mirabekov et al. used another method to develop protein chips. They immobilized small polyacrylamide gel pads on a glass surface and immobilized oligonucleotides, DNA, various types of proteins such as antibodies, antigens and enzymes on to this microchip[7-9]. Zhu et al. have used nanowell technology to produce microwell chips in silicon elastomer sheets. The chips assayed kinase – substrate reactions in the microwells by first immobilizing the substrate using an amino cross linker. They used radio labeling for detection[9].

Substrate libraries have played an important role in determination of substrate specificities with regards to proteases. Proteases play a central part in all biological cycles with its ability to selectively cleave specific substrates, which regulate various biological functions within an organism. The specificity of the substrate is relational, it depends on the concentration of the protease and its co – factors, its inhibitors; with regards to its spatial position and the preceding events. The specificity of the substrate is however finally determined by the active sites present on the protease. Thus determination of the substrate specificity can help us elucidate and provide a deeper perspective in the role proteases plays in the biological pathways[10-12]. The libraries can be generated synthetically or biologically. Biological substrate libraries have been developed on filamentous phage[10-13] as well as E. Coli.[14] to determine protease specificity. Ellman et al. devised a positional scanning combinatorial library where all the 20 amino acids can be used at the P1 position and allows for rapid analysis of substrate specificity using synthetically generated fluorogenic substrate libraries[11,12].

II. AIMS AND OBJECTIVES

We have decided to develop a new platform technology which simultaneously achieves: (1) the full compatibility of inexpensive glass slides with standard instrumentation; (2) liquid phase reactions with minimal evaporation; (3) the absence of cross-contamination; and (4) ability to rapidly assemble multicomponent reactions. This platform should enable us to perform microarray based enzyme assays, which use fluorogenic substrates to test the enzymatic reactions that take place in complex biological fluids. The objective is to

develop a technology that will help in studying protein function, specifically related to enzymes. Enzyme assay microarrays can assist in phenotyping blood [16-18]. Blood represents an ideal tissue for *functional proteomics*: (1) Blood is easily obtained; (2) many mutations are known with associated bleeding or clotting phenotypes; (3) coagulation protein knockout mice are available; (4) the protein biochemistry, reaction network complexity, and cell biology of blood are well characterized; and (5) blood clots that cause heart attacks and strokes remain the highest source of mortality in the US. Enzyme assay microarrays can serve a role in blood phenotyping, as well as in clinical diagnostics or bedside coagulation monitoring.

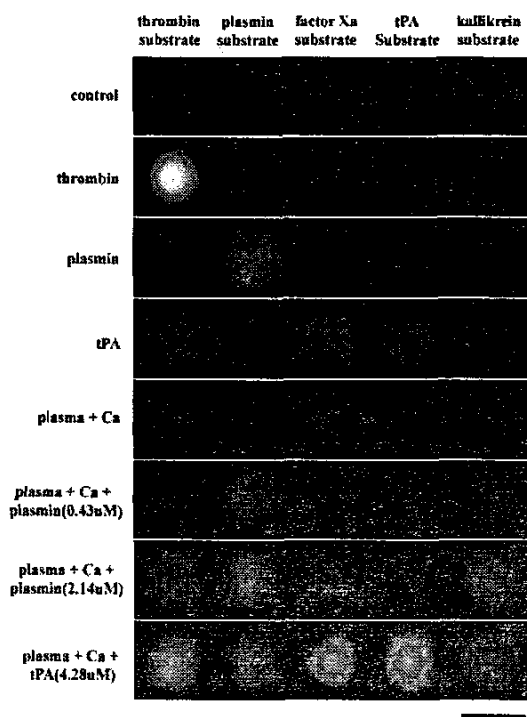


Fig.1. Microarray based assay of purified enzymes and human plasma. Replicate five spot arrays for sensing protease activities for thrombin, plasmin, factor Xa, tPA, and kallikrein were activated by spray deposition of various solutions. Exposure of the array to control buffer without enzyme demonstrates the low background of the unconverted substrate (row 1). Microarrays were activated with buffer containing 1U/ml thrombin (row 2), 1 μ M plasmin (row 3), and 10 μ M tPA (row 4) or activated with human recalcified citrated platelet free plasma alone (row 5) or activated with a final concentration of 0.43 μ M plasmin (row 6), 2.14 μ M plasmin (row 7), or 4.28 μ M tPA (row 8). Bar = 400 μ m.

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