

HIGH-THROUGHPUT MULTIPLEXED PROTEASE ACTIVITY MEASUREMENT USING A DROPLET BASED MICROFLUIDIC PLATFORM WITH PICOINJECTOR

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ABSTRACT

In this study, we integrated several components, including a droplet generator, a pico-injector, and an analytical inference technique, Proteolytic Activity Matrix Analysis (PrAMA), to create a platform for assessing multiple specific protease activity assays with minimal liquid handling and sample-requirement for personal medicine analysis. The microfluidic platform enables the direct measurements of protease enzyme activity, which is more physiologically informative than the standard measurements to determine the enzyme concentration alone. By tracking hundreds of picoliter droplets containing biological samples mixed with unique FRET-based protease substrates and inhibitors, the assay simultaneously infers multiple specific protease activities with minimal (<20 μ l) physiological sample requirement. We applied this method to *in vitro* study of an immortalized cell line established from a peritoneal endometriotic biopsy to ascertain the proteolytic activity response of TNF- α treatments.

KEYWORDS

Multiplexed activity bioassay, Droplet based technique, Protease, Emulsions, Picoinjector

INTRODUCTION

Proteases play essential roles in several physiological and disease processes. In particular, matrix metalloproteinases (MMPs) and A Disintegrin and Metalloproteinases (ADAMs) have been investigated as potential diagnostic biomarkers for cancer metastasis and endometriosis [1]. Existing approaches such as zymography, activity-based enzyme-linked immunosorbent assays (ELISAs), and activity-based probes have been limited by trade-offs including throughput, simultaneous measurement of multiple activities (multiplexing), and direct kinetic measurement. Alternatively, an analytic method, Proteolytic Activity Matrix Analysis (PrAMA) was developed for ascertain of multiple proteolytic activities of MMPs and ADAMs [2]. The approach of PrAMA provides the possibility to elucidate particular enzyme activities from cleavage signatures obtained in clinical samples containing multiple proteases. However this analytical method requires massively multiplexed measurements, which usually requires large amount of clinical samples to cause the difficulties in real applications of diagnosis. Although the analytical methods for high sensitivity measurements of protease activity was investigated [3], multiplexed protease activity assay remain challenges. To address this issue, we introduce a method to combine PrAMA with a droplet based microfluidic platform to simultaneously determine multiple (nine different kinds of combinations, ~1000 individual reactions), specific protease activities from small amounts of physiological samples (<20 μ L). The picoinjector [4] provides programmable controls of adding reagents into the droplets for large scale multiplexing assay by using small amount of sample volume, while the PrAMA provide a method to analyze the massive results obtained from the picoinjector for searching the protease biomarker effective. Moreover to achieve the automatic assay, a program was developed to analyze large number of different reactions within the droplets simultaneously to accelerate workflow and throughput of experiment for statistical confidence. We analyzed physiological samples, an immortalized cell line (12Z) established from a peritoneal endometriotic biopsy, to ascertain the proteolytic activity response of TNF- α treatments.

EXPERIMENTAL RESULTS AND DISCUSSION

The device shown in the schematic diagram (Fig.1A) was fabricated as a Polydimethylsiloxane (PDMS) chip bonded to a PDMS-coated glass slide. The flow rates of aqueous/oil streams into the device were controlled by using syringe pumps (PHD 2000, Harvard Apparatus, Holliston, MA). To

develop the multiplexed assay, the protease substrate library was first formed using droplet generator chips [5] which contain a flow-focusing geometry with fluorocarbon oil as the carrier fluids. The droplets encapsulated different fluorogenic protease substrates and were stored in a large tube as a droplet library [6]. To distinguish the type of substrate in the droplets, a specific concentration of indicator dye was added as an optical label. In the second step, the droplets were uploaded (Fig.1B) to a picoinjector device where they were added to the biological samples (Fig.1C). The DC voltage was applied to disturb droplet stability by changing surfactant arrangement on the surface of droplets and thus the sample solution (containing enzyme, protease) could be injected into droplets (containing substrates) for enzyme activity reactions. The flow rate of the injection was controlled as $\sim 0.03 \mu\text{L}/\text{min}$ and the DC voltage applied was $\sim 80\text{V}$ with frequency of 1kHz . The droplets were subsequently sent to a large chamber for time-lapse observation of reaction progress (Fig.1D). The protease reactions within droplets were analyzed by the increases of product fluorescence. With the advantage of automated image analysis, the reaction rates from ~ 1000 droplets were obtained simultaneously (Fig.1E), and the reaction rates of different substrates in the droplet library were analyzed with high a degree of statistical confidence (Fig.1F).

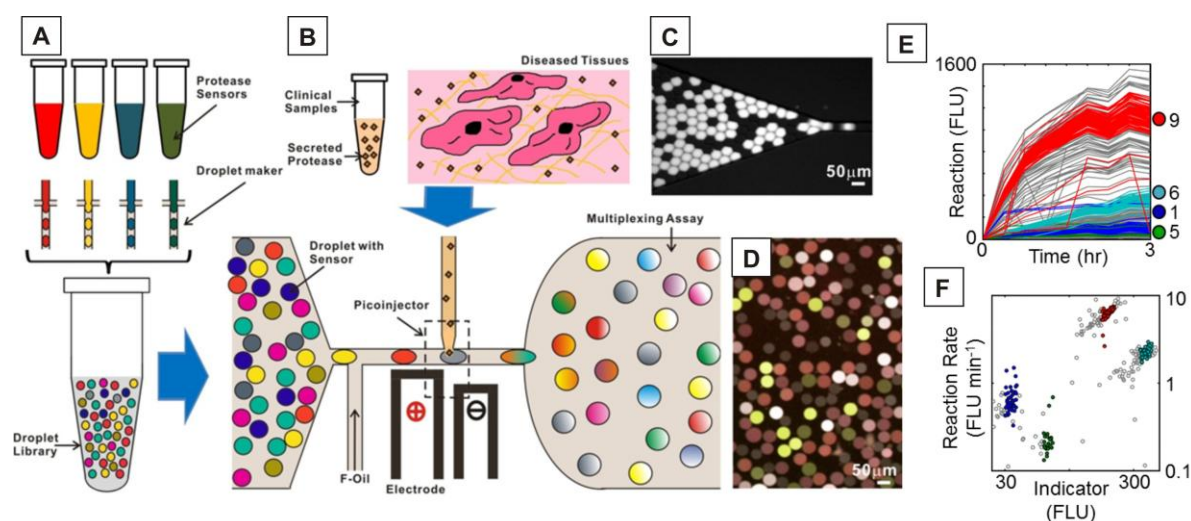


Fig.1: (A) Schematic representation of the work flow and microfluidic device was represented. (B) All droplets were uploaded into a picoinjector device with high volume fraction order. (C) The droplets were passed through the picoinjector device for the sample addition into the droplets (D) The reactions in droplets were analyzed by a program automatically with high throughput manner in a large observation chamber. (E) The droplets with different substrates (1,5,6,9) were analyzed automatically through a software program.(F) The type of substrates was identified by the intensity of indicator fluoresce (Alexa-546, wavelength 546nm)

We first tested the ability of microfluidic PrAMA to identify an individual enzyme based on the pattern with which the panel of substrates in the droplet library reacted with the enzyme. To demonstrate, we constructed a 4×4 parameter matrix describing the cleavage efficiencies of four protease substrates (1,5,6,9) by recombinant MMP7 & 9 and ADAM10 & 17 at 1.0 nM , respectively (Fig.2A, left). We compared patterns of catalytic efficiency with those measured on a plate-reader, and observed high correlation between the microfluidic and standard assays ($R^2 > 95\%$). We then used PrAMA to infer specific enzyme activity based on the observed pattern of substrate cleavage. Using the protease activity measurements from individual enzymes as a training set, PrAMA demonstrated an ability to identify individual enzymes based on their cleavage profile with 99% accuracy (Fig.2A, right). As a test set, we then employed PrAMA to infer specific enzyme activities from a mixture containing both ADAM10 and ADAM17. Based on the observed rates of substrate cleavage in the droplet library (Fig.2B, left), PrAMA correctly inferred the presence of ADAM10 and ADAM17 with $>99\%$ specificity (Figure2B, right). As a second demonstration, we used device to infer protease activities in cellular supernatant secreted from treated/control cells (Fig.2C). Experiments show substrate-9 reacts strongly with treated supernatant, and PrAMA infers upregulated ADAM9 activity from the altered pattern of substrate cleavage (Fig.2D).

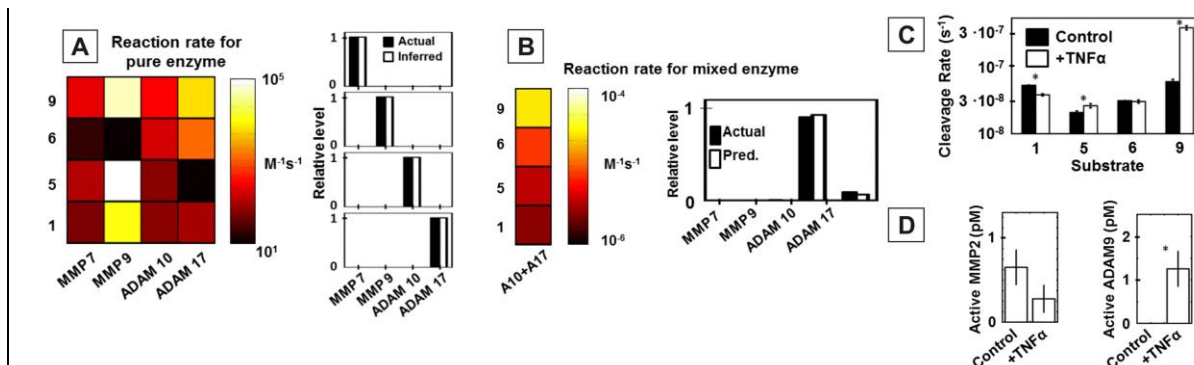


Fig.2 (A) Hierarchical biclustering of observed cleavage efficiencies for various ADAMs and MMPs were tested (left). The results were consistent by the standard plate-reader (right) for the confirmation. (B) The reaction activities of mixed enzyme and substrates were characterized by their corresponding cleavage signatures (left). The composition of active mixed enzyme therefore could be determined (right). (C) The cleavage of TNF α cellular supernatant and substrates was tested. Compared with the control sample, the treated cells secreted more proteases to react with substrate-9. (D) The reactions show the treated cells secreted more ADAM-9 than the control ones.

CONCLUSION

In summary this work integrated several components, including a droplet generator, a pico-injector, and an analytical inference technique (PrAMA) to create a platform for assessing multiple specific protease activity assays with minimal liquid handling and sample-requirement. Accomplishing the same multiplexed measurements in a 96-well microtiter plate would consume at around 100-fold more biological sample and reagent. It is also noteworthy that only a tiny fraction of 20 μ l sample volume was actually utilized to generate droplets for multiplexed sensing, due to unoptimized world-to-chip interfacing. This leaves much room for efficiency improvement, either by drastically increasing the degree of multiplexing or further decreasing the sample volume needed. The microfluidic platform thus enables the direct measurement of protease enzyme activity, which is more biologically informative than measurement of the enzyme concentration alone. The modularity among the droplet based microfluidic technology, and the methods to determine multiple enzyme activities ultimately makes the platform highly customizable for potential applications in high-throughput personal medicine analysis and clinical detections

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