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Single cell multiplexed assay for proteolytic activity using droplet microfluidics

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ABSTRACT

Cellular enzymes interact in a post-translationally regulated fashion to govern individual cell behaviors, yet current platform technologies are limited in their ability to measure multiple enzyme activities simultaneously in single cells. Here, we developed multi-color Förster resonance energy transfer (FRET)-based enzymatic substrates and use them in a microfluidics platform to simultaneously measure multiple specific protease activities from water-in-oil droplets that contain single cells. By integrating the microfluidic platform with a computational analytical method, Proteolytic Activity Matrix Analysis (PrAMA), we are able to infer six different protease activity signals from individual cells in a high throughput manner (~100 cells/experimental run). We characterized protease activity profiles at single cell resolution for several cancer cell lines including breast cancer cell line MDA-MB-231, lung cancer cell line PC-9, and leukemia cell line K-562 using both live-cell and *in-situ* cell lysis assay formats, with special focus on metalloproteinases important in metastasis. The ability to measure multiple proteases secreted from or expressed in individual cells allows us to characterize cell heterogeneity and has potential applications including systems biology, pharmacology, cancer diagnosis and stem cell biology.

1. Introduction

Multiple enzyme activities in individual cells correlate to a series of cellular processes determining their biochemical state, function and fate; even seemingly identical cells with the same genotype can exhibit strikingly distinct behavior due to alternative post-translational regulation of tightly interconnected enzyme networks (Kovarik and Allbritton, 2011). As a result, multiplexed biochemical measurements at single cell resolution, particularly when those measurements directly measure activity and catalytic product formation, are valuable for revealing the true biochemical state and behavior of biological samples. Metalloproteinases including matrix metalloproteinases (MMPs) and a Disintegrin and Metalloproteinases (ADAMs) represent a particularly important family of highly regulated enzymes that have gained attention for their prominent roles in physiological and pathological processes related to extracellular matrix remodeling, cellular migration, and extracellular signaling. Consequently, metalloproteinases have been increasingly investigated as potential drug targets and diagnostic biomarkers of disease. Many studies have reported that an up-regulation in MMP/ADAM activities can cause an increase in the invasiveness of individual cancer cells including progression of the epithelial-mesenchymal transition (EMT) (Radisky and Radisky, 2010; Orlichenko and Radisky, 2008); cleavage of cellular surface biomarkers (receptors) (DeLano and Schmid-Schonbein, 2008); and release of apoptotic ligands (Van Lint and Libert, 2007) due to their ability to degrade various proteins of the extracellular matrix (Moulik et al., 2014). However, most current multiplexed bioassays follow ensemble approaches that study average behavior across cell populations (Huang et al., 2013; Yan et al., 2011; Lowe et al., 2012), thereby lacking the ability to address heterogeneity across individual cells (Irish et al., 2004). Although many technologies including flow cytometry and microscopy allow single-cell measurements of various molecular markers to be performed, it has been difficult to measure the actual real-time catalytic activities of specific proteins rather than mere presence or concentration (Miller et al., 2011; Ramji et al., 2014).

Several micro fabricated platforms have been investigated for single cell analysis. For example, micro-valve technology was

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demonstrated to perform different applications of single-cell intracellular measurements with a throughput of ~100 cells per experimental run (Lu et al., 2015; Wu et al. 2015). The capillary electrophoresis system was automatized to analyze ~3.5 cells/min (Dickinson et al., 2013; Fan et al., 2008; Di Carlo and Lee, 2006). Compared with these methods, droplet based microfluidics is attractive because of its ultra-high throughput (~1000 droplets/s) and accurate fluidic controls that enable precise fluidic mixing and cell encapsulation (Ramji et al., 2014). Accordingly, a wide range of droplet-based single cell polymerase chain reactions (PCR) have been successfully carried out for DNA/RNA analysis in previous studies (Novak et al., 2015; Macosko et al., 2015; Romero et al., 2015; Chattopadhyay et al., 2014; Herderschee et al., 2015). Secreted protein concentrations have also been quantified in single-cell droplet assays (Deng et al., 2014; Abbaspourrad et al., 2015, Jing et al., 2015), but without the ability to simultaneously analyze multiple analytes (multiplexing) and without the ability to observe actual enzymatic activity. Thus, although powerful, these past studies have failed to capture many post-translational regulatory events that are critical to understanding overall cellular behaviors and their dynamics.

In this study, FRET (fluorescence resonance energy transfer)-substrates were modified to accommodate different fluorescent pairs with distinct excitation and emission wavelengths to obtain multiple signals of enzymes from single-cell encapsulated droplets. We focused on analyzing multiple metalloproteinase activities, and used the computational approach Protease Activity Matrix Analysis (PrAMA) to translate FRET-substrate cleavage measurements into specific enzymatic activity read-outs (Miller et al., 2011). Individual MMP/ADAM cleavage efficiencies were quantitatively ascertained using purified recombinant enzymes. PrAMA then used these measurements as standards to mathematically infer specific enzyme activity levels from complex biological samples. Droplets containing single cells were immobilized in an observation chamber and were analyzed for time-lapse reaction screening over the course of 2 h.

Fluorescent signals contributed by the protease-substrate reactions in a single cell droplet were measured simultaneously to obtain specific protease activities. With the advantages of PrAMA, we were able to infer the activities of six key MMPs and ADAMs from four FRET-based peptide substrates. As a proof of principle, we tested the platform on breast cancer cells (MDA-MB-231), lung cancer cells (PC-9) and leukemia cells (K-562). The platform was performed in two complementary formats that demonstrated single-cell measurements from both cell lysate and live cell cultures. The capability of this assay to resolve single cell heterogeneity brings new perspective in cancer characterization that is unattainable through bulk analysis. Ultimately, the ability to determine multiple protease activities at single cell resolution has the potential to provide network-level insight into enzyme regulation, and could be used to characterize rare individual cells such as highly malignant circulating tumor cells in cancer patients.

2. Material and methods

2.1. 1 Experimental methods

The microfluidic device was fabricated using standard polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning Inc., Midland, MI) methods (Supplementary-1). The MDA-MB-231 (ATCC), PC-9 (IBL) and K-562 cell lines (ATCC) were cultured in RPMI (for PC-9) and DMEM (for MDA-MB-231 and K-562) supplemented with 10% FBS and penicillin-streptomycin (Supplementary-2). All substrates were prepared by diluting the four lyophilized substrates into PBS (1 ×) at a concentration of 10 μM each. The four substrate peptide sequences were selected from a previously characterized library (Supplementary-3), and were selected such that the activities of six MMPs and ADAMs of high interest (MMP-2, MMP-3, MMP-9, ADAM-8, ADAM-10 and ADAM-17) could be distinguished and determined with high sensitivity and selectivity. Substrate UV was selected to identify ADAM activities (especially ADAM-17); substrate B was selected to identify MMP activities (especially MMP-2 and MMP-9); substrate G was used to separate MMP-9 activity from MMP-2 activity; and substrate R was used to identify ADAM-8 activity.

Fig. 1 demonstrates the experimental method. Suspended cells were first passed through a pinch-flow channel to mix with multiple modified FRET-substrates for cell encapsulation in water-in-oil droplets (Fig. 1a and b). For cell lysis, 0.5% Triton-X was used to permeate cell membranes, and was added to the mixture of substrates before loading the mixed solution into a microfluidic chip. For cell encapsulation within droplets, aqueous streams of cells and substrates were mixed, at which point an oil-phase stream was introduced to sheer the mixed aqueous flow into droplets at the co-flow channel using syringe pumps (Harvard, PHD2000). The oil flow rate was set at 5 μL/min while both aqueous flow rates were set at 2.5 μL/min to form ~10 pL droplets with a generation rate of ~4 kHz. Fluorocarbon oil HFE 7500 (3M NovecTM, Singapore) with 0.5% krytox (modified) surfactant was used to generate stable, monodisperse 40 μm droplets *via* the droplet generator microfluidic chip. The droplets were then sent to an observing chamber, where time-lapse fluorescence signals (Supplementary-4, Supplementary-MV1) were monitored under fluorescence microscopy and a sCMOS camera (Hamamatsu Orc2).

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Four substrate-protease reactions (in UV, Blue, Green and Red fluorescence channels) in a droplet were simultaneously monitored at four distinct pairs of fluorescent excitation (maximum excitation UV: 400 nm, B: 490 nm, G: 546 nm, R: 635 nm) and emission (maximum emission UV: 420 nm, B: 520 nm, G: 580 nm, R: 670 nm) wavelengths (Fig. 1c). Substrate UV, B and G were excited by using Nikon Intensilight C-HGFI Mercury Lamp (Nikon, Japan) at 365 nm, 470 nm and 546 nm, while substrate R was excited using the automated excitation system (CoolLED pE-2) with bandpass filter and emitter wavelengths of 635 nm. Microscope optical filters were used to minimize crosstalk of signals for these multiplexed measurements (Fig. 2). The raw fluorescent signals were processed by subtracting the average intensity of empty droplets from the intensity of droplets encapsulating cells. Image J software was used to process images and analyze droplet intensities. The bright field image of the droplets was recorded to ensure single cell encapsulations in droplets. Empty droplets and droplets with multiple cells were excluded from this analysis. Fluorescence increase over time was normalized to its peak fluorescent intensity (obtained from full cleavage of all substrates by Trypsin-EDTA) for each substrate. The normalized reaction rates were compared with the plate reader to ensure accuracy of the readout (Supplementary-5).

2.2. Substrate calibration

To ensure that different signals could be analyzed in a droplet containing an individual cell, fluorescent bleed-through between substrates was examined. The optimal concentrations for substrates were characterized to be 10 μM (Supplementary-6). After full cleavage by trypsin, the excitation and emission spectra of each substrate were examined using a microtiter plate fluorimeter (Fig. 2a), and results showed only minimal cross-talk in substrate fluorescence.

To further prevent fluorescent cross-talk, optical filters