High-throughput, Microfluidic Monitoring of Enzymatic Activity for Personalized Medicine

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Abstract:
Medical treatments are often highly generalized across patients with a particular disease. Treatment and drug efficacy become hampered as a result due to inherent heterogeneity between patients and between diseases. Therefore, to optimize therapeutic effects and minimize hazardous side effects, a personalized approach to medicine, involving individually tailored treatment plans (i.e. type and dosage of therapeutic), is desired. This requires preliminary tests, known as companion diagnostics, to assess the efficacy of a drug prior to administration. For cancer treatment, there is a growing interest in targeting differential enzymatic activity with prodrugs (precursor forms activated by an intracellular enzyme). Understanding enzymatic activity of the tumor prior to prodrug administration will allow physicians to design a better therapeutic plan. Currently, however, there exists no such method. Therefore, as a tool for personalizing cancer treatment, we have developed a novel, robust technology to accompany all prodrug treatments. This technology, termed "P-MED" (**P**ersonalized **M**onitoring of **E**nzyme **D**ynamics), provides quantitative enzymatic activity measurements of single cells in a high-throughput manner by monitoring the turnover of fluorogenic substrate in a microfluidic device. Cells are manipulated using inertial effects that occur on the microscale to allow synchronized measurements of fluorescence accumulation that report on enzymatic activity. We have used this system to measure intracellular esterase activity (currently targeted for colorectal cancer therapy) in proof-of-concept model cells (Jurkat and HeLa cells). Additionally, P-MED can monitor the response to enzymatic inhibitors (nordihydroguaiaretic acid). P-MED can be used to predetermine the success of a variety of drugs on a per-patient basis. This method has far reaching implications in cancer treatment and pharmaceutical drug testing.

Introduction:
Current medical treatments, including cancer treatments, are unable to achieve optimal therapeutic efficacy across a wide range of patients due to over-generalizations during initial drug testing and development[1]. Cancer is a highly prevalent disease with a high death rate; more than 1,500 people die of cancer per day within the United States. Despite recent efforts to personalize therapies, cancer treatments are largely generalized with standard practices and dosages applied to most patients. This approach cannot combat the inherent variation within tumors and across patients[1]. The most common form of treatment, following resection of large tumor masses, is chemotherapy. This treatment is used to non-selectively destroy cancer cells at the cost of harming healthy cells in the patient. Current treatments do not address the complexities in the field of cancer care[2–10].

Drug efficacy can be highly variable due to patient heterogeneity. Such complexity necessitates a personalized approach to medicine, which involves understanding and administering therapy on an individualized basis. Companion diagnostics are tests designed to assist physicians in making treatment-related decisions by defining expected responses to various therapy and treatments based on patient-specific data. These tests can help predict outcomes prior to drug administration[2-6].

Personalized medicine is an emerging field butt there are very few companion diagnostics currently available, with most of these tests focused on identifying mutations leading to drug sensitivity or resistance in a cancer[3,11]. One exciting area of drug therapies that has not been personalized yet makes use of an inactive form of a drug that requires specific enzymatic action to become active. These drugs, called prodrugs, are a subclass of chemotherapeutics that would benefit from preliminary assays that evaluate drug conversion in cancer tissues[12]. Additionally, there has been a growing push to take advantage of differential enzymatic activity with prodrugs. Widely researched enzyme/prodrug combinations such as esterases/Irinotecan herpes simplex 1 virus thymidine kinase/ganciclovir, cytosine deaminase/5-fluorocytosine, carboxypeptidase G2/CMDA, and horseradish peroxidase/indole-3-acetic acid have demonstrated promising early clinical results, but are hindered by a lack of knowledge of target differences in enzymatic activity between cancerous and healthy tissue[12,13].

There is a need to develop a general tool to monitor the efficacy of prodrugs or to understand if a prodrug is a strong candidate prior to administration[8–10]. Such a tool could impact a wide range of fields and populations, as well as become a standardized tool for pharmaceutical companies to test their drug on an individual patient's

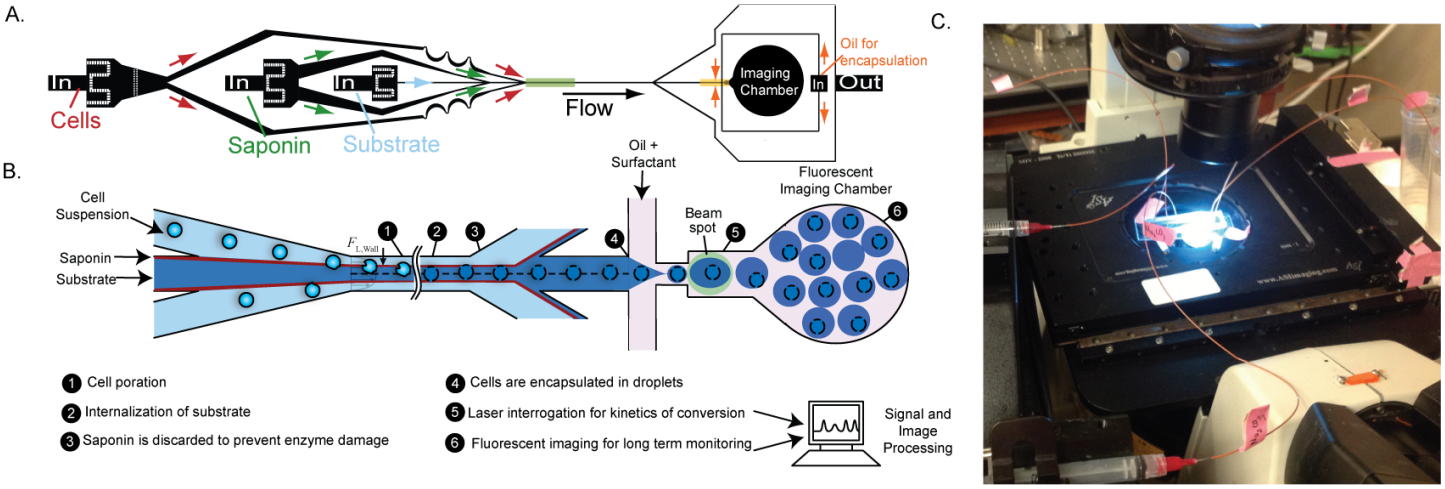

Figure 1. Prototype of final device. *A. Computer-aided design (CAD) of the device. Photolithography and replica molding techniques are used in device manufacturing. Device footprint is a few inches. B. Cartoon schematic of device operation. Cells are introduced through an inlet and are passively manipulated across a stream of Saponin (a non-ionic detergent that disrupts membranes), and finally into a solution containing the substrate for the enzymes of interest. The quick exposure of Saponin prevents cell death. Cells are then packaged into droplets for fluorescence measurements. C. Image of device mounted onto a microscope for monitoring operations. Syringe pumps are used to drive fluids through the device.*

cells to provide per-patient care. Measurements on a single cell level, rather than bulk blended tissue, must be made because bulk measurements of heterogeneous populations can mask the presence of important subpopulations of cells for targeted treatment[14–16]. Additionally, measuring the intracellular activity requires operating at throughputs that combat the complexity of biological samples.

Current tools to monitor enzyme activity are lacking. For example, they may be able to monitor numerous single cells but delivery of impermeable fluorogenic dyes as enzyme substrates into the cell often is difficult. Additionally, these systems are not fully integrated and automated. Substrate internalization, activity measurements, and analysis are decoupled. Also, numerous methods fail to have a simple output to record activity (e.g. fluorescence)[17–20].

In this report, we present a high-throughput, robust system ("P-MED" – **P**ersonalized-**M**onitoring **E**nzyme **D**ynamics) to measure intracellular enzymatic activity to provide diagnostic information to caregivers. This technology utilizes shifts in inertial forces in microfluidic channels to manipulate cells[21–23]. Cells are introduced into the system and are manipulated across a porating stream to briefly permeabilize them to allow for internalization of impermeable fluorogenic enzyme substrate. Next, the cells are introduced to the enzyme substrate and are subsequently packaged for interrogation and automated analysis using a MATLAB script (Figure 1A-B). A final device setup is shown in Figure 1C. This technique can monitor the activity of hundreds of single cells at multiple time points to provide a deep understanding of the enzymatic activity within tumors.

Materials & Methods:

Overview: For our system to be useful, we needed to characterize and develop several components. First, the microfluidic components for substrate delivery and long-term enzymatic monitoring were developed. Next, methods and scripts for automated analysis were developed. Preliminary characterization of the system was performed by monitoring cellular activity of various cells and also monitoring cellular response to enzymatic inhibitors.

Device Fabrication and Operation. The microfluidic devices used in this process were created from polydimethylsiloxane (PDMS; Sylgard 184 Silicone Elastomer Kit; Dow Corning Corp.) as previously described [19]. Standard photolithographic methods and replica molding techniques were used as previously described for all processes.

Polyether ether ketone tubing (PEEK; Upchurch Scientific) was connected on one end to syringes with 25-gauge luer stubs (Instech Laboratories, Inc.). The free ends of the tubing were connected to the inlets of the

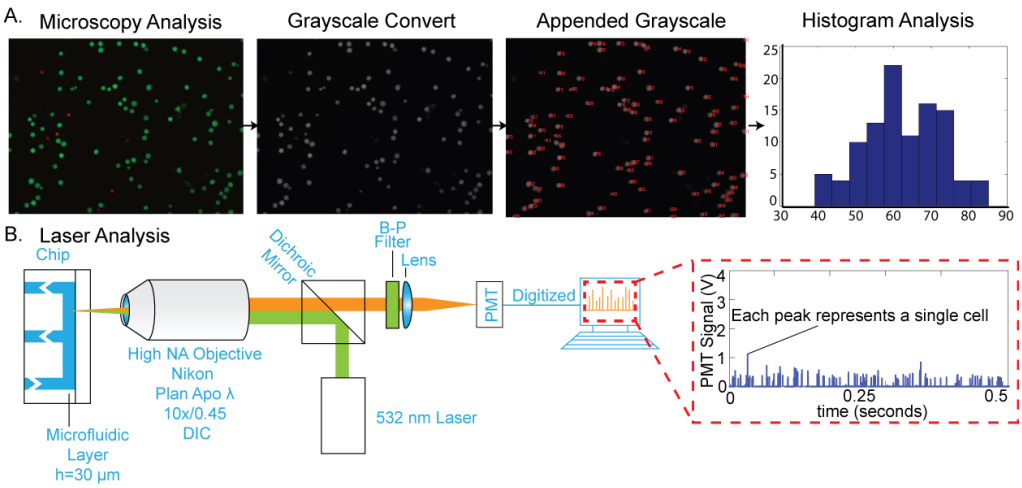

Figure 2. Two Modalities of Monitoring Enzymatic Activity: Long and Short Term. *A. Cells that have internalized substrates are collected and imaged with fluorescence microscopy to acquire long term metabolism data. Fluorescent micrographs of single-cells are analyzed with our custom MATLAB script, which converts images to grayscale to measure intensity of each cell. Each cell is located by thresholding images to form a binary image. Images can be appended with fluorescence intensity measurement for further corroboration into histograms or evaluation by clinicians. Additionally, we developed numerous quantitative tools for data analysis and can present all this information in easy to read formats. B. Flow Cytometry Setup. Microfluidic device functionalized with a 532 nm laser interrogation scheme to analyze inertially focused single cells for short-term enzymatic activity acquisition. The Photomultiplier Tube (PMT) band-pass filters the data and presents the digitized data, where each individual peak represents a single cell with varying enzymatic activity passing through the beam spot.*

device, which was mounted on an inverted Eclipse Ti microscope (Nikon) coupled to a high-speed camera (Figure 1C; Phantom v7.3 high-speed camera, Vision Research Inc.).

Droplet Generation System. An alternate device design was developed to encapsulate cells after exposure to substrate solutions. We used 19 μm polystyrene beads (Bangs Laboratory) as a model for cells in fluid flow. Beads were encapsulated by surfactant stabilized Mineral Oil, Heavy (Fisher Scientific) and transferred to a Phosphate Buffer Saline (PBS; Thermo Scientific) solution. Oil was perfused at 15 μL/min concurrently with beads at 40 μL/min and PBS at 60 μL/min.

Automated Image Processing with a Custom MATLAB Script. We developed a custom MATLAB script capable of analyzing images of fluorescent cells and quantifying intensity associated with fluorescence. The script uses built-in MATLAB functionality to convert the fluorescent color image to a binary and a grayscale image (Figure 2A). Individual cells are identified with the binary image function and their grayscale intensity is quantified. The program is designed to append these values directly to the grayscale image to allow for comparison with manual measurement programs such as ImageJ. Importantly, the program provides histogram plots of fluorescence intensity to determine each individual cell's fluorescence, which is correlated with enzymatic activity.

Laser Detection System. Jurkat cells were incubated with Calcein AM (532 nm excitation) prior to introduction into an alternate straight microfluidic channel. A continuous-wave laser (Laser Quantum) set at 532 nm measured fluorescence intensity on the single cellular level. The data was band pass filtered as it was acquired, and collected by a photomultiplier tube (PMT). The digitized and peak finding algorithm was applied and further analyzed by a custom MATLAB code developed to characterize these intensity peaks. Data was measured at 1 MHz for 1 second with a 50 μm beam spot (Figure 2B). An inline system such as this will enable the study enzyme kinetics.

Poration Feasibility, Cellular Viability, and Detecting Differences in Cellular Activity. Ethidium Homodimer (Invitrogen), 1% Saponin (Sigma-Aldrich), and Jurkat cell (ATCC TIB-152) solutions were filled into separate syringes and introduced simultaneously at constant volumetric flow rates with PHD 2000 Syringe Pumps (Harvard Apparatus) into the device at 120 μL/min, 15 μL/min and 50 μL/min, respectively. Collected cells were then transferred into a 96 well plate where Calcein AM (Invitrogen) was added. Cells were subsequently imaged on a fluorescent microscope with a Coolsnap HQ fluorescent camera (Photometrics) to confirm successful poration, internalization of Ethidium Homodimer, and maintained viability.

To determine differences in cellular activity, a Calcein AM solution replaced the Ethidium Homodimer solution used for internalization. 4 minutes after collection, cells were imaged four times in 2-minute increments using

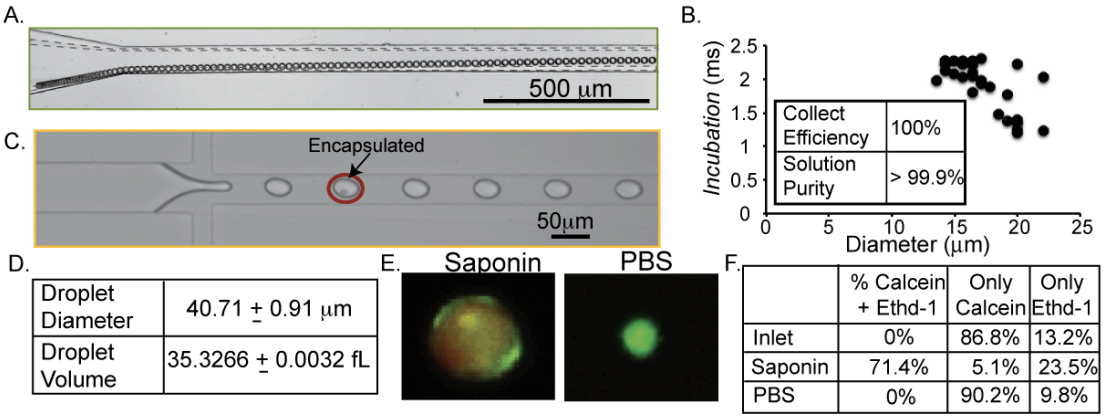

Figure 3. Characterization, operation, and demonstration of fluidic components. *A. Overlaid images of a high-speed camera operating at ~8000 frames/sec. A single microparticle is travelling through the poration section of the device. B. Characterization of incubation time in the Saponin stream. Incubation time is tuned to be very short (a few ms) to prevent cell death. The Saponin stream width can be tuned easily to alter incubation times as needed. C. Image of droplet generation portion of fluidic device (corresponds to yellow box region in Fig. 1A). D. Characterization of droplets reveals stable and uniform droplet production. (μm = 10^{-6} meters, fL = 10^{-15} liters). E. Impermeable substrates are delivered to cancer cells. Representative image of a porated cell successfully stained with Ethidium Homodimer (red, impermeable DNA dye) as well as Calcein (green, indicates viable cell) at F. high efficiency (71.4%). No internalization in the control with PBS validates chemical, not mechanical poration.*

fluorescence microscopy. The cells were imaged once again at 15 minutes post collection. Changes in fluorescence intensity were quantified with ImageJ (National Institutes of Health) as well as an automated MATLAB script (The MathWorks, Inc.).

Esterase Inhibition. Nordihydroguaiaretic acid (NDGA; Cayman Chemical) solutions were prepared at 1 *n*M, 1 μM, and 100 μM with constant concentrations of Calcein AM (3 μM). Porated and substrate-containing cells were imaged with fluorescence microscopy at 5, 10, 20, 30, and 40 minutes after collection. Images were analyzed for fluorescent intensity on ImageJ and our automated MATLAB script.

Results & Discussion:

Microfluidic Component Characterization. After developing the integrated microfluidic platform (Figure 1) and the two modes of fluorescence detection and analysis (Figure 2), we worked to characterize the device. Consistent monitoring of enzyme activity and internalization of non-permeable substrates necessitates that all cells are exposed to homogeneous poration conditions[7]. We demonstrated that our device could successfully transfer cells and particles across the fluid poration stream (Figure 3A). The timescale for incubation of cells in porating solution was measured. We found that the incubation time was approximately 2 milliseconds with little variation over a range of sizes, validating the ability to expose all cells to homogeneous conditions (Figure 3B). However, the conventional time scales of Saponin incubation (1-2 hours) according to standard manufacturing poration protocols do not match our rapid incubation times. To account for this, Saponin concentrations were increased to achieve poration on millisecond time scales (1% w/v in PBS). We also measured the device efficiency in transferring particles (diameter = 20 μm) across the fluid streams and collecting them to be 100% and verified that there was very little cross-solution contamination (solution purity > 99.9%).

We developed a method of monitoring enzymatic activity for long time periods by encapsulating the substrate-containing cells in water-in-oil droplets. After solution exchange, cells approach a T-Junction through which oil is flowing such that it causes a pinching that results in a droplet. We also characterized this component of our system. We found that successful encapsulation of particles is feasible in our integrated system for substrate delivery and analysis (Figure 3C). Additionally, we confirmed that the droplets generated were monodisperse (low standard deviation) and large enough in size to carry a single cell. Droplet diameter was 40.71 μm ± 0.91 μm. Droplet stability was determined by observing changes in volume over a period of time and looking for shearing of droplets in expanding channels. The droplet volume remained constant and did not shear with channel expansion, suggesting that the droplets would remain stable for long term (minutes to hours) monitoring. Importantly, the standard deviation was small (+/- 0.02%) to ensure uniform reaction control.

Demonstration of Inline Analysis. Successful interrogation of cells while simultaneously traveling through P-MED allows for a supplementary method of fluorescence analysis to long term monitoring. This method proved

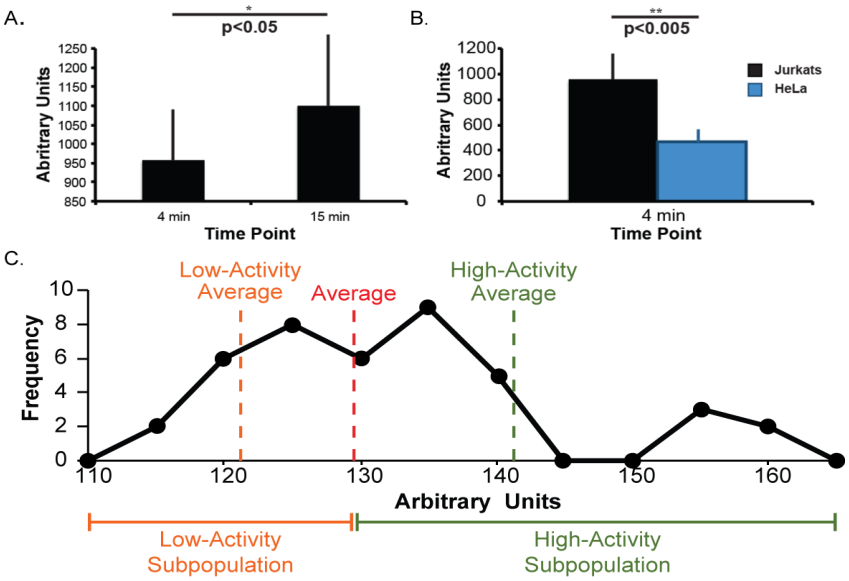

Figure 4. Identification and differentiation of tumor cell types. *We used our system to differentiate and identify tumor cells by measuring intracellular esterase activity. A. Long-term monitoring of fluorescence turnover of Jurkats for understanding how metabolism of a prodrug will occur in vivo. B. Jurkat and HeLa cell esterase activity. HeLa cells have significantly lower esterase activity than Jurkat cells. This information can be used by clinicians to differentiate between tumor types when administering drugs (e.g. Irinotecan). C. Single-cell analysis enables identification of subpopulations within a tumor sample. By identifying these low-activity subpopulations, a clinician can choose a particular drug regime to attack this population to make sure that all tumor cells are eradicated.*

successful in capturing each cell's intensity reading, as indicated by the number of individual readings gathered.
Figure 2B shows the output of a photomultiplier tube, where each peak represents a cell passing through a laser beam spot. Jurkat cells were perfused in a straight microfluidic channel at the optimal flow rates that allowed proper inertial focusing of cells and ordering such that the beam spot would interrogate one cell at a time. Over a hundred cells were detected in one second of interrogation. Therefore, this method of cellular interrogation was developed in order to ensure both short term and long term monitoring could be feasible with P-MED. The ability to perform inline measurements provides enhanced functionality to this system, now enabling study of rapid dynamics. We were able to pick up small differences in fluorescence intensity using this inline system. The ability to detect small differences enhances the statistical accuracy of our system when identifying cell populations of interest for directed prodrug targeting.

Characterizing Uniform Cell Poration and Substrate Internalization. To verify consistent poration and substrate internalization, Jurkat cells (T lymphocytes) were processed through P-MED. Jurkats were manipulated across a stream of Saponin (within a few milliseconds) to reach a final solution of Ethidium Homodimer (Ethd-1). Ethd-1 is an impermeable, high-affinity nucleic acid stain that fluoresces when it binds to DNA. Therefore, if cells were successfully porated, they would be positive for Ethd-1. Cells were uniformly porated upon brief exposure to Saponin, enabling substrate internalization. Furthermore, cells were labeled with Calcein-AM after they exited our device to verify retained enzyme function. Of the cells collected, a distinctly defined nucleus (red, Ethd-1) can be seen with preserved esterase activity (green, Calcein AM) (Figure 3E). 71.4% of cells processed through P-MED were positive for both Ethd1 and Calcein AM showing high-efficiency poration without damaging enzymatic activity. This indicates the potential to deliver to cells a variety of therapeutically useful, membrane impermeable substrates—such as fluorogenic prodrug-analogs. Additionally, preservation of enzymatic activity enables accurate monitoring of drug metabolism, which can help tailor a physician's treatment from patient to patient. We performed some controls to ensure that we were performing successful chemical poration and not harming the cells in another manner. Replacing the middle incubation solution with phosphate buffered saline (PBS), a harmless buffer, resulted in 0% poration (0% cells were + for both Ethd-1 and Calcein) (Figure 3E-F).

Distinguishing Enzymatic Activity Differences and Cell Subpopulations. We demonstrated that our system could distinguish enzymatic activity between different cell types by using esterases as a model enzyme. Esterases are especially interesting as they can act to cleave ester groups on Irinotecan, a commercialized prodrug for colorectal cancer. There, however, is no companion diagnostic associated with Irinotecan. Previously, it has been shown that Jurkat cells exhibit an increased conversion of Calcein AM (a fluorogenic dye that requires ester cleavage to become fluorescent) in comparison to HeLa cells[20]. We first tested our ability to track the dynamics of substrate conversion. Jurkat cells were introduced into P-MED and Calcein AM was introduced. Calcein AM fluoresces upon cleavage from intracellular esterase. We were able to track fluorescence increases over time. Fluorescence increased as more of the Calcein AM was cleaved (957.2 $\pm$ 141.9 A.U. at t = 4 min compared to 1099.6 $\pm$ 192.2 A.U. at t = 15 min., $p<0.05$) (Figure 4A). We also

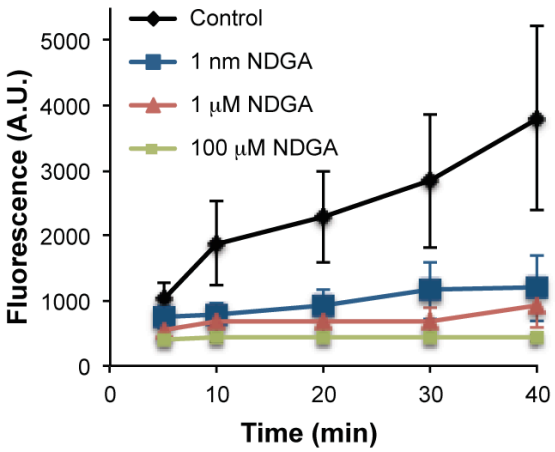

Figure 5. Monitoring drug response and understanding dosage effects. *Esterase inhibitors and substrates were delivered to Jurkats using P-MED. Esterase activity was measured with varying concentrations of inhibiotrs. We successfully monitored drug response. This information can be useful in determining the effective dosage of a drug needed to have a therapeutic effect.*

monitored the esterase activity of HeLa cells. We were able to distinguish the activity of these two cells types. As expected, HeLa cells had a lower esterase activity in comparison to the Jurkat cells (Jurkat 957.2 A.U. compared to HeLa 468.2 A.U. at t = 4 min., $p<0.005$) (Figure 4B). The ability to quantify differences in enzymatic activity between subpopulations of cells confirms that P-MED could be used to gather information about patient tumors prior to drug treatment.

We additionally modeled heterogeneity within cell populations. Jurkat and HeLa cells were spiked together at equal concentrations in one solution with Calcein-AM and processed using P-MED. In Figure 4C, there is a subpopulation of cells that have lower metabolic activity (110-120 A.U.), which most likely correspond to HeLa cells due to their lower activity as shown in Figure 4B. There is also a subpopulation of cells that are more metabolically active (150-160 A.U.), which most likely correspond to Jurkat cells from their higher average enzyme turnover rate. This shows that P-MED is able to detect differences in fluorescence intensity in mixed populations of cells and can potentially be applied to clinically relevant samples to assay optimal drug treatments. The presence of two subpopulations further motivates the need for single cell measurements, as taking an average fluorescent intensity would completely mask the apparent subpopulations present.

Detecting Differences in Enzyme Activity through Varying Substrate Concentration. To demonstrate the ability of P-MED to process substrate concentration dependence, an esterase inhibition was also carried out using nordihydroguaiaretic acid (NDGA). We have shown that increasing the concentration of NDGA causes increased inhibition of esterase ability to cleave Calcein AM. Jurkat cells exposed to 1 nM NDGA after 40 minutes exhibited 1197 A.U. of fluorescence; cells exposed to 1 μM NDGA exhibited 945 A.U.; cells exposed to 100 μM NDGA exhibited 421 A.U. (Figure 5). These values in comparison to the 40-minute time point for the control (3799 A.U.) demonstrate the concentration dependence of esterase inhibition by NDGA. Models of drug effects can be formed by using P-MED to run samples at subsequent time points over a relatively long period of time (days to months) to monitor drug effects in a patient. This might be of great importance to a physician as dosage amounts of Irinotecan can be tailored by understanding the effective concentration of the drug that is needed to have a significant impact.

Conclusion:

Here we present a novel, rapid method to measure intracellular enzymatic activity. Our innovative system for personalized medicine, termed P-MED, aims to understand the cellular metabolism of prodrugs prior to treatment administration. It addresses numerous challenges, such as developing easy-to-quantify indicators of enzymatic activity at the single-cell level to report on intracellular enzymes. P-MED utilizes inertial forces in microfluidic channels to manipulate cell migration across different types of solutions. This enables cell poration, internalization of fluorogenic substrates that normally cannot cross the membrane, and monitoring of their activity. P-MED operates at high-throughputs, making it possible to process and analyze individual cells at rates greater than 1000 cells/sec, which helps addresses tumor heterogeneity. We have also been able to detect differences in cell populations using a model enzyme system, with P-MED's ability to perform single-cell analysis enabling detection of low and high activity subpopulations. We have also developed two imaging modalities for the development of this translational tool: short term in-line laser detection and long term fluorescent microscopic observation. Short and long term monitoring allow for acquisition of substrate metabolism to achieve a broader understanding of enzyme activity. Both of these methods are directly interfaced with custom software to simplify data collection and analysis for physicians. With the future integration of these components, we envision the widespread adoption of P-MED to accompany drug testing and any prodrug treatment.

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