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Catching Villains: Finding single cells responsible for cancer drug resistance and metastasis

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Disclaimer

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Catching Villains: Finding single cells responsible for cancer drug resistance and metastasis

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Abstract

Despite the widespread acceptance that cell-to-cell variability is a defining feature of cancer, much remains unknown about stem-cell like cancer cells that are hypothesized to be responsible for both metastasis and drug resistance (Marusyk, Almendro, and Polyak 2012). Few analytical techniques exist that can quantify single cell drug uptake, hindering progress towards decoding how cancer cells evade therapeutics. This project aims to address the dearth of analytical chemical methods available for single cell analysis, by employing accelerator mass spectrometry (AMS) to directly quantify chemotherapeutic uptake in single cells. *We have successfully built a platform to measure single cells with Lawrence Livermore National Laboratory's (LLNL) biological AMS instrument and developed new methods to further fractionate cell contents which could be used in conjunction with AMS to quantify drug action.*

Using our platform, we demonstrate correlation between single cell measurements and populations averaged means with an R^2 of 0.93. Next, we measured single breast cancer cells treated with carboplatin, a first line chemotherapeutic, after 4, 12 and 24 hours of exposure. We found some evidence of a small population of cells present at 12 hours that had a higher amount of drug, suggesting that bulk averages may not accurately describe the drug uptake in this system.

We chose to focus on droplet microfluidics (Brouzes et al. 2009, Guo et al. 2012) as a platform technology to fractionate of single cells, with the ultimate goal of applying these methods to isolate drug adducts from single cells to quantify drug action. Using a microfabricated array of traps, droplets were formed and used to extract DNA from single cells. Efficiencies of 70-90% were achieved for various steps of this process, which involved initial droplet formation with droplets containing cells, beads, and lysis buffer, incubation of droplets to allow cell lysis to occur, "breaking" of the droplets to enable a wash step, and re-formation of droplets to maintain the integrity of single cell DNA upon removal of droplets from the device.

Background and Research Objectives

As evidence mounts that cellular heterogeneity within a population is critical in defining both healthy and disease states, reliable techniques for chemical analysis at the single-cell level are critical. Cancerous tumors are notorious for being heterogeneous, and only trace amounts of cancerous cells are required for drug resistance and metastasis. However, little research has been done to examine drug exposure at the single cell level. Fluorescent optical reporters used to label proteins and biomolecules has greatly advanced our understanding of cancer biology, but coupling optical probes to small molecule pharmaceuticals significantly alters their structure and can affect their function and metabolism. On the other hand, radiolabeling pharmaceutical drug molecules generates chemically identical analogs, however direct chemical analysis of single cells remains out of reach for most techniques due to the exceedingly small amounts of chemotherapeutics at the single cell level. Therefore, we sought to **fill the void of techniques capable of directly quantifying drug uptake in single cells, by developing a platform to *reliably* deliver single cells to LLNL's exquisitely sensitive biological AMS system, enabling full realization of AMS' potential to investigate single-cell heterogeneity.**

We believe AMS will complement other types of mass spectrometry methods used to investigate intracellular components, such as MALDI-TOF (Zhang and Vertes 2015) and electrospray

ionization mass spectroscopy (ESI-MS). ESI-MS methods use ambient ionization, allowing cells to remain in near-native states (Baumeister et al. 2019; Huang et al. 2011), and can identify up to hundreds of metabolites from a single cell (Duncan, Fyrestam, and Lanekoff 2019). However, these methods are generally restricted to high abundance species, and quantification is reliant on internal standards. Methods such as nanoSIMS (Legin et al. 2014) and inductively coupled plasma-mass spectroscopy (ICP-MS) (Corte Rodríguez et al. 2017) take advantage of the signature of platinum-based chemotherapeutics to demonstrate differential chemotherapeutic uptake in single cells. Unfortunately, these methods do not work with drugs that do not contain an easily identifiable group (*e.g.*, platinum). Isotope labeling provides an alternative method that does not significantly alter the drug's structure and allows drug quantitation using mass spectroscopy. *Of note, AMS offers direct quantification at extremely low zeptomole levels not possible with other methods* (Rubakhin et al. 2011; Lapainis, Rubakhin, and Sweedler 2009; Zenobi 2013; Urban et al. 2010)*; this sensitivity will allow us to detect important metabolites, such as drug-adducts, that are below the limit of detection for other techniques (**Fig. 1**)* (Enright et al. 2016; Rubakhin et al. 2011; Lapainis, Rubakhin, and Sweedler 2009; Zimmermann et al. 2017; Henderson et al. 2011; Wang et al. 2010; 2016; Hah et al. 2006).

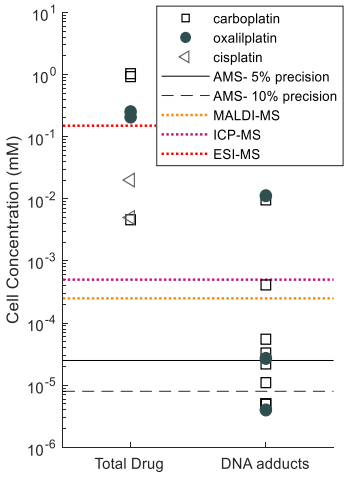

Figure 1. *AMS has the sensitivity to quantify platinum chemotherapeutics in single cells, unlike other techniques. Lines represent the minimum single-cell concentration for 5 and 10% precision in AMS measurement and limit of quantification for other available chemical analysis techniques. Markers are estimated single-cell platinum chemotherapeutic concentrations in whole cells (total drug), and for DNA-drug adducts based on reported values of bulk drug measurements.*

We used carboplatin, a first line chemotherapeutic, commonly used to treat various cancers including bladder, lung, ovarian, colon and prostate. The drug acts by covalently modifying cellular DNA, inducing cell death. Recent work suggests that only measuring drug accumulation in cells alone (rather formation of DNA-drug adducts) may not predict drug susceptibility (Wang et al. 2016). Therefore, we further developed novel methods to manipulate single cells and perform biological assays on chip. Although the potential for learning from studies on single cells (particularly from heterogenous cell populations such as those from a tumor) has been widely acknowledged, technology to enable isolation and analysis of single cells lags in development. Several commercial products exist (i.e. from nanocellect N1 cell dispenser, ALS-Jena's CellCelector, and Eppendorf's Transferman), there are significant limitations to these technologies, including their high cost, low throughput, and in some cases, a high degree of skill or training required to operate the equipment.

Microfluidics is a platform technology to automate biological assays, and has been applied to high-throughput studies of single cells. Wells, partitions, or droplets may be used to isolate individual cells to a small volume, although each approach imposes limitations on the applications and downstream analysis of the captured cells. Well-based isolation strategies offer the potential for fluid exchange, which could enable multi-step assays (such as DNA or protein isolation from the cells), but analysis of results is limited to *in situ* observation of well contents, which cannot be removed from the device (Marcus et al. 2006). Droplets are also commonly used for single cell analysis (Brouzes et al. 2009, Guo et al. 2012), and have even been applied to multi-step assays (Gu et al. 2011, Stephenson 2018), however the throughput of these systems is low owing to the fact that droplets are processed one-by-one. With this type of workflow, each additional assay step requires a complete re-working of the microfluidic device architecture, and often requires active controls to synchronize reagent addition to droplets and prevent unwanted droplet collisions. Furthermore, this style of processing prevents dynamic observation of droplet contents during and following the assay.

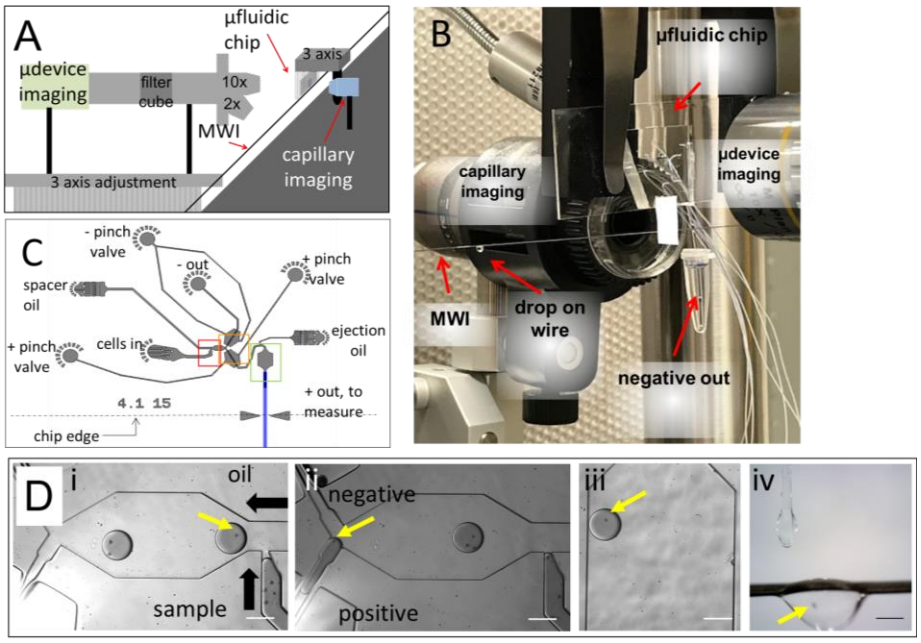

Figure 2. *Developed Platform. A) Schematic of the sample delivery platform coupled to the MWI, including imaging at both the wire and in the µfluidic chip. Not shown are fluid delivery systems including both syringe and pressure pumps, pressure control systems for elastomeric pinch valves on chip and analog output signals generated to align imaging data with AMS results. B) Picture of a µdevice positioned over the MWI. C) CAD representation of the µfluidic chip. D) Sequential images of a single cell being sorted and delivered to the MWI. Scale bars are 100 µm for images inside the µfluidic chip, and 500 µm for the picture of the capillary and moving wire (3). Yellow arrows point to the cell and droplet being sorted and ejected.*

We have designed a microfabricated trap array to allow *in situ* generation and exchange of fluid within the droplets. This workflow provides benefits of both the well-based and the droplet-based microfluidic single cell analysis platforms, allowing both droplet retrieval for downstream analysis as well as parallel processing and dynamic observation of droplet contents during an assay.

Scientific Approach and Accomplishments

Single Cell AMS

We have created a microfluidic platform that enumerates, sorts and delivers single cells to our bio-AMS moving wire interface (MWI), the means by which liquid samples are introduced into the accelerator (***Fig. 2***). First, cells are singulated and encapsulated in aqueous-in-oil droplets to facilitate manipulation and minimize and standardize incompatible and confounding components of suspension media such as salts, sugars and residual ^{14}C signal. The singulated cell solution is injected directly into the sorting chip, and single cells are encapsulated by co-flowing oil, to facilitate manipulation (***Fig. 2Di***). Using elastomeric pinch valves (Sundararajan, Kim, and Berlin 2005), droplets with single cells are directed to a holding chamber where cells are further visually verified (***Fig. 2Dii,iii***). While deformable microfluidic valves are slower to actuate than electrophoretic sorting mechanisms, they are well suited for isolating specific chambers, and allow us to repeatably eject cells from the chip and deposit them on the MWI (***Fig. 2Div***). Our previous attempts to measure single cells involved labor-intensive cell picking using a micromanipulator and microinjector to withdraw individual cells into a micropipette tip, and process the entire tip as graphite. Cell picking was limited to 1 cell/15 minutes followed by graphite conversion which takes multiple days. In contrast, using the developed system, we successfully measured cells at rates of approximately one cell per three minutes.

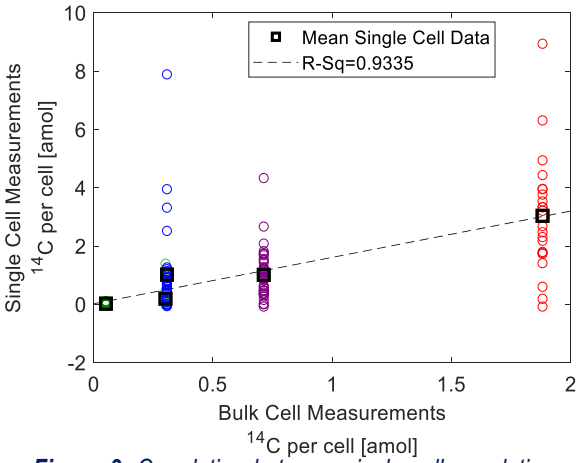

Figure 3*. Correlation between single cell populations and bulk measurements. Cells were radiolabeled with varying degrees of 14C, and measured at the single cell level using the developed platform (colored markers). 14C content was quantified in bulk cell samples (~104 cells) via graphite AMS analysis.*

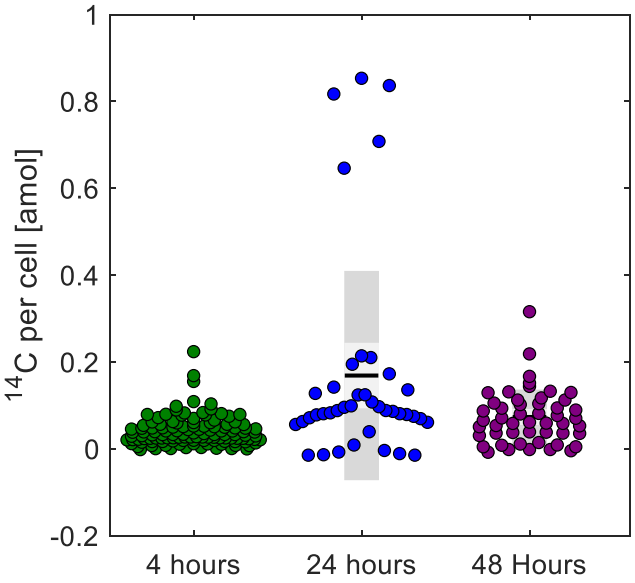

Figure 4. *Distribution of carboplatin uptake in MDA-231 cells after 4,24 and 48 hours. N=122,40,33, respectively.*

We quantified whole-cell ^{14}C content in individual cells cultured in media supplemented with [^{14}C]thymidine media using this platform (***Fig. 3***). The single cell data is well correlated with bulk measurements of the same cells (~10^4 cells) measured using the standard procedure, R^2=.93. We expect that continued refinement of the platform and further investigation of the linear measurement range will improve the correlation further. We suspect the large variation within similarly treated cells is largely due to biological heterogeneity due to differences in cell size and cell state, and have previously seen similar behavior in yeast cells cultured in media supplemented with [^{14}C]glucose.

Next, we measured MDA-MB-231 breast cancer cells treated with radiolabeled carboplatin. This is a model cell line for invasive, triple negative breast cancer and is commonly used to study drug resistance. Since the ^{14}C signal is exclusive to the carboplatin, the amount of ^{14}C detected in each cell can be used as a proxy for drug absorption. We dosed cells for 4, 24 and 48 hours the drug concentration expected to kill 50% of the population by 48 hours. At 4 hours, we see very low

amounts of drug in nearly all cells. We expect that this is due to minimal drug being absorbed at this early time point. At 24 hours, we see evidence of a small population of cells which appear to be high in drug, which are not present at 48 hours. This data may indicate that this population high in drug concentration at 24 hours were susceptible to the drug, and lysed by 48 hours. During measurement, we select for whole cells, thus any cells which have died and lysed will not be measured. Our results demonstrate the ability to quantify drug uptake in single cells, and potentially identify important subpopulations that would not be detectable with population averaged measurements.

DNA Extraction

We have developed a microfluidic platform that can form and trap droplets containing single cancer cells and perform multi-step fluidic exchange toward execution of a complete workflow for DNA extraction. The platform (***Fig. 5a, b***) and workflow (***Fig. 5c***) are shown below. Droplets are formed *in situ* by design of microfabricated structures that enable compartmentalization of an aqueous phase containing cells, beads, and assay reagents. To form droplets, an aqueous phase is initially infused into the microfluidic device. Cells contained in this aqueous phase are retained by hydrodynamic trap features designed to permit flow of fluid through the trap until a cell enters and blocks flow. This design minimizes the number of traps containing multiple cells. Once traps are fully occupied with the aqueous phase containing cells, an immiscible oil phase (Novec HFE 7500 fluorocarbon oil, 3M) with or without surfactant (Pico-Surf, Sphere Fluidics) is infused to the device. Owing to the interfacial tension between the aqueous and oil phases and the capillary pressure valves created at the small (30 μm) openings at the back of the traps, oil is not permitted to enter the trap structure as it fills the device. This process forms one droplet per trap structure, containing the cells and beads initially infused with the aqueous phase. Subsequently, a second aqueous phase can be infused and under the right conditions, the emulsified droplets will "break" enabling the aqueous trap contents to mix with the second infused aqueous phase. ***This step is critical for multi-step assays where new reagents must be added, solutions mixed, and wash steps executed, and***

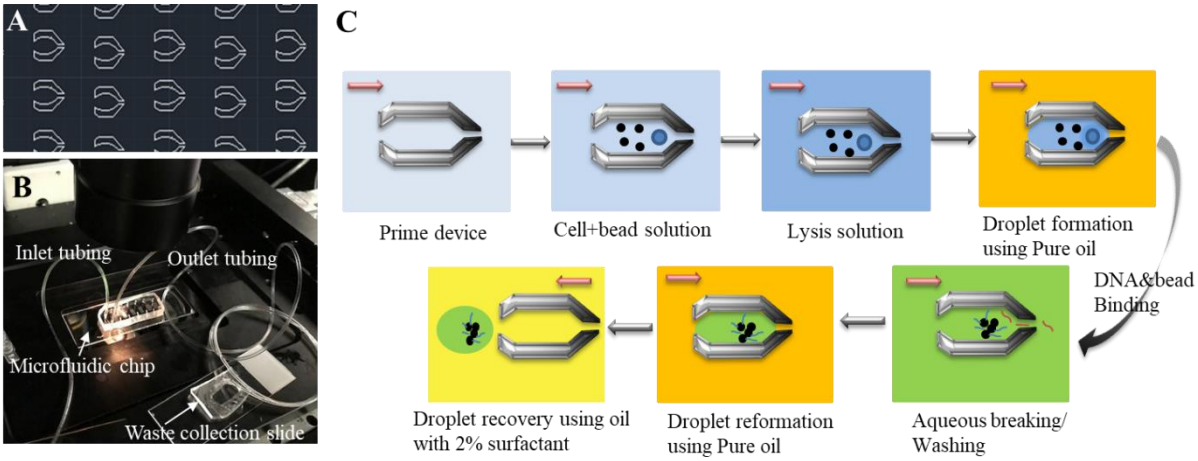

Figure 5. *Experimental setup. (A) Portion of chip design of the arrayed traps. (B) Instrumentation for a microfluidic experiment. (C) Schematics for the complete experiment workflow to enable single cell DNA extraction. First, beads and cells are incorporated into traps using a plug flow within the tubing. Following this step, a lysis buffer along with pure oil phase is infused in a separate plug to generate droplets at a high flowrate of 50,000 uL/hr to minimize cell lysis before droplet formation. Following droplet formation, incubation time allows for DNA to bind to the encapsulated beads, after which the emulsion is broken by infusion of a second, washing, aqueous phase which carries away cell debris that are not bound to the beads and pure oil is infused to facilitate droplet reformation. The reformed droplets are recovered by flowing 2% surfactant backward by gradually increasing the flowrate from 500 uL/hr to 5000 ul/hr.*

overcomes a major limitation of droplet microfluidics technology. Importantly, the droplets formed in this device can be recovered off-chip for subsequent analysis in the AMS, other spectroscopic instruments, or other applications involving sequencing.

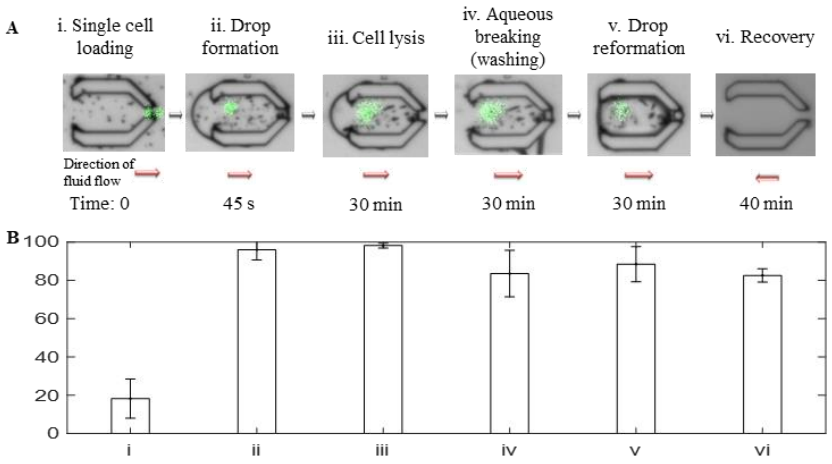

Figure 6*. On-chip DNA extraction using the array traps. (A) Cells and DNA binding beads were loaded into the traps, followed by lysis buffer and oil to form droplets. Cells were allowed incubate until lysis was visually observed. Following this step, the droplets were broken using wash buffer to remove unwanted cellular residues. Droplets were then reformed by introducing oil and then droplets containing the extracted DNA bound to beads were recovered by gradually increasing the flow rate of oil with surfactant to stabilize the droplets during removal. (B) Success rates for subsequent steps of the assay (i-vi) presented in A.*

The steps for DNA extraction on the microfluidic device are as follows:

(1) Fill traps—Infuse beads and cells in aqueous solution
(2) Infuse lysis buffer
(3) Form droplets – Infuse fluorocarbon oil phase to form droplets *in situ* at the trap structures
(4) "Break" emulsion – infuse second, washing aqueous phase
(5) Droplet re-formation – infuse second fluorocarbon oil phase to form droplets with washed contents
(6) Droplet recovery—infuse fluorocarbon oil phase from the opposite direction to dislodge drops from traps and recover in a separate device outlet

Design of the microfluidic trap structures was modified to include a taper at the back and front of the trap, which facilitated droplet formation and enables droplets to remain trapped in the absence of flow (i.e. for incubation steps of an assay). Using the modified array trap device, droplets could be generated at an occupancy rate of 87% (step 3), trapped droplets could have their fluid exchanged and were then able to reform droplets at a rate of 48% (step 5), and then recovered from the device at a rate of 84% (step 6). When using 100 μL cell solution of 1E5 cells/mL samples, 9-13% of single cell per trap occupancy was observed.

After optimizing conditions for droplet generation, fluid exchange, and recovery, the traps were used to perform DNA extraction of 4T1 cancer cells (***Fig. 6***). DNA was extracted using the Dynabeads DNA Direct Universal extraction kit (ThermoFisher), with optimized buffers. Off-chip buffer optimizations were performed to achieve a high purity of the DNA extract (characterized by having a 260/280 ratio greater than 1.8). The lysis/bead binding buffer contains 0.06 M NaCl; 0.03 M Tris-HCl pH 8; 0.003% SDS; and 2.4 M guanidine hydrochloride (GnHCl) in DI Water. The washing buffer 1 is composed of 9 mM Tris-HCl pH 8, 0.135 M LiCl, and 0.6 M GnHCl in DI Water, and washing buffer 2 contains 10 mM Tris-HCl pH 8, 0.15 M LiCl in DI water. Following extraction, DNA was fluorescently stained with Quant-iT^{TM} PicoGreen Reagent for imaging demonstrations.

In summary, using the array traps we demonstrated that DNA extraction of single cells can be performed on chip. The trap array allows positioning of cells at a predetermined location and enables monitoring of each cell within the trap. Cell lysis is depicted in Figure 6A where the fluorescent intensity disperses upon cell lysis when the DNA is no longer enclosed in the cell membrane. If necessary, this device can also facilitate mixing within each trap using magnetic beads and magnets.

Impact on Mission

This project has leveraged LLNL's Center for Accelerator Mass Spectroscopy (CAMS) unique Bio-AMS, and resulted in a capability to directly quantify single cells using with AMS, that will further boost LLNL as the authoritative figure in the field of Biological AMS. This project lays the groundwork to significantly expand the applications of AMS for single cell analysis. The Center for Accelerator Mass Spectroscopy (CAMS) at LLNL hosts the NIH-funded National Center for Research Resources for Biomedical Accelerator Mass Spectrometry as a resource frequently used by investigators from all over the world, and we expect this capability will be of interest to many investigators.

This project has generated significant interest with collaborators in the UC Davis Comprehensive Center Consortium (UCDCCC), and resulted in the award of a joint pilot grant from UCD to Dr. Steve George and Drs. Erika Fong and Ted Ognibene. This engagement with the UCDCCC is critical to engaging with experts in fields that LLNL doesn't have specific expertise in and having access to clinical samples.

Through this project, we were able to hire Hawi Gemeda into a post-bachelor position. Hawi completed her Masters Project with Dr. Melinda Simon, a collaborator on this project at San Jose State University (SJSU). She spent a few months working on this project as a summer student; bringing the cell array technology from SJSU to LLNL, and further developed methods and techniques to complete cell assays on chip at the single cell level. Following her graduation, Hawi started working full time at LLNL during the winter of 2018. She has continued to do great work on this project, as well as contributing to significant efforts across engineering, specifically performing experiments at the interface of additive manufacturing and biology. She has been a strong addition to the LLNL family, and will continue to contribute to the progress made by the lab.

Through collaboration with SJSU throughout the project, we have gained access to an additional avenue for microfluidic device design, testing, and fabrication, as well as a pipeline of bachelor's- and master's-level graduates trained in these skill areas. Master molds for the sample delivery platform and droplet trap array were fabricated in the Microscale Process Engineering Laboratory (MPEL) at SJSU, and the Simon lab has offered continued assistance in fabricating additional master molds and microfluidic devices for the continuing work on this project. In addition, five of Dr. Simon's master's students continue work on aspects of the project. Two of these students continue the work on DNA extraction in the microfabricated trap arrays, with the aim to collect additional data demonstrating the biological relevance of the platform. They seek to publish this work in summer 2020. Three of these students are continuing work to demonstrate proof-of-concept DNA extraction on a parallel technology platform (digital microfluidics) that would enable more precise manipulation of droplets for assays involving additional steps or greater wash purity (e.g. mRNA, rather than genomic DNA extraction).

Conclusion

In this project we have developed two novel microfluidic systems to 1. generate a new capability to quantitate single cells using AMS, and 2. perform biological assays on single cells, such as DNA

extraction. We demonstrate good correlation between our novel single cell measurements of radiolabel uptake with the current state of the art bulk techniques. We further show that our single cell droplet platform can be used to perform complex biological assays at the single cell level to isolate DNA from individual cells. We expect that this work will serve as preliminary data necessary to get further NIH funding to investigate single cell populations, and are actively pursing open calls. Due to the universal nature of radiolabeling, these technologies can be applied to numerous biological systems, from the direct quantification of pharmaceuticals to fundamental cell metabolism research.

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Publications and Presentations

1. Erika Fong, Melinda Simon, Nick Hum, Ted Ognibene, and Gabriella Loots. "Empowering Accelerator Mass Spectrometry to Find Single Cells Responsible for Cancer Drug Resistance." presented at the 5th Annual Southern California Micro and Nanofluidics Symposium, Arcadia CA, August 24, 2018.
2. Erika Fong, Melinda Simon, Nick Hum, Ted Ognibene, and Gabriella Loots. "Empowering Accelerator Mass Spectrometry to Quantify Single Cell Chemotherapeutic Uptake." presented at the SelectBIO World Congress: Single Cell Analysis Summit 2018, Coronado CA, October 2, 2018.
3. Erika Fong, Nick Hum, Kelly Martin, Bhup Shergill, A Anand, V Shirure, Ted Ognibene, and Steve George. "Quantifying Drug Delivery to Single Tumor Cells in a 3D in Vitro Model of the Tumor Microenvironment." presented at the 25th Annual Cancer Research Symposium, Sacramento, CA, September 2019.
4. Young, E., Dodati, C., Fong, E., Simon, M.G. "Evaluation of Biofouling of Hydrophobic and Omniphobic Coatings for Digital Microfluidic Chips." presented at the Biomedical Engineering Society (BMES) 2019 Annual Meeting, Philadelphia, PA, October 2019.

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