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UNIVERSITY OF CALIFORNIA SAN DIEGO

**A single-cell assay and a biosample enrichment method for analyzing single
cell secretion**

A dissertation submitted in partial satisfaction of the
requirements for the degree
Doctor of Philosophy

in

Materials Science and Engineering

by

Wei Cai

Committee in charge:

Professor Yu-Hwa Lo, Chair
Professor Yi Chen
Professor Louise Laurent
Professor Tse Nga Ng
Professor Liangfang Zhang

2019

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The Dissertation of Wei Cai is approved, and it is acceptable in
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University of California San Diego

2019

DEDICATION

To David Lawrence Hall

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Chapter 4 is based on and mostly submitted papers: Wei Cai, Yi-Huan Tsai, Edward Wang, Ping-Wei Chen, Lennart Langouche, Chuan Lu, Tao Wei, Yu-Hwa Lo, “Bioparticle enrichment using mass transport via IR-induced differential evaporation rate” and Wei Cai, Edward Wang, Ping-Wei Chen, Yi-Huan Tsai, Lennart Langouche, Yu-Hwa Lo, “A microfluidic design for desalination and selective removal and addition of components in biosamples”. The dissertation author was the primary investigator and author of this paper.

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PUBLICATIONS

1. Yu-Jui Chiu, **Wei Cai**, Yu-Ru V. Shih, Ian Lian, and Yu-Hwa Lo, A single-cell assay for time lapse studies of exosome secretion and cell behaviors, **Small**. 12, 3658-3666 (2016). DOI: 10.1002/smll.201600725
2. Yu-Jui Chiu, **Wei Cai**, Yuesong Shi and Yu-Hwa Lo, A self-confined single-cell loading platform combining PDMS mesh and patterned Cytop for non-invasive studies of single cell secretions. 2016 **IEEE 16th International Conference on Bioinformatics and Bioengineering**. DOI: 10.1109/BIBE.2016.15
3. Yu-Jui Chiu, **Wei Cai**, Tiffany Lee, Julia Kraimer and Yu-Hwa Lo, Quantitative Analysis of Exosome Secretion Rates of Single Cells. **Bio-protocol**. 7(4): e2143 (2017). DOI: 10.21769/BioProtoc.214
4. **Wei Cai**, Yu-Jui Chiu, Valya Ramakrishnan, Yi-Huan Tsai, Clark Chen, Yu-Hwa Lo, A single-cell translocation and secretion assay (TransSeA). **Lab on a Chip**, 18 (20): 3154-3162, (2018)
5. **Wei Cai**, Yi-Huan Tsai, Edward Wang, Ping-Wei Chen, Lennart Langouche, Chuan Lu, Tao Wei, Yu-Hwa Lo, Bioparticle enrichment using mass transport via IR-induced differential evaporation rate
6. **Wei Cai**, Edward Wang, Ping-Wei Chen, Yi-Huan Tsai, Lennart Langouche, Yu-Hwa Lo, A microfluidic design for desalination and selective removal and addition of components in biosamples

ABSTRACT OF THE DISSERTATION

A single-cell assay and a biosample enrichment method for analyzing single cell secretion

by

Wei Cai

Doctor of Philosophy in Materials Science and Engineering

University of California San Diego, 2019

Professor Yu-Hwa Lo, Chair

One of the key challenges of biology is to understand how individual cells process information and respond to perturbations. However, most of the existing single cell analysis methods can only provide a glimpse of cell properties at specific time points and are unable to provide cell secretion and protein analysis at the single cell resolution. This thesis offers the description of a single-cell assay as well as a CO₂-induced enrichment method for the analysis of single cells secretions.

The single-cell assay introduced in this thesis enables the accommodation of different cellular types, allows for easy and efficient single cell loading and culturing, and is suitable for studying the effects of in-vitro environmental factors in combination with drug screening. One salient feature of the assay is the non-invasive collection and survey of single cell secretions at different time points, producing unprecedented insight of single cell behaviors based on the biomarker signals from individual cells under given perturbations. In addition, the open-well design of the assay allows for simple collection of cells with standard tools such as pico-pipette for downstream processes in relating the single-cell secretions with gene analysis. Above all, the acquired information is quantitative. For example, measured by the number of exosomes each single cell secretes for a given time period, exosomal miRNA carried by exosomes secreted by single cell. Therefore, this single-cell assay provides a convenient, low-cost, and robust tool for quantitative, time lapsed studies of single cell properties.

Another challenge for single cell secretion analysis is the limit-of-detection (LOD) and sensitivity. Thus, sample enrichment is an important step in the work flow of biosensing for disease detection and numerous biological or clinical processes. Most current techniques require devices that are tailored to specific chemical or physical characteristics of the target objects to enrich or capture them from the sample. The complexity within these devices all serve to, increase cost and may even limit the enrichment factor. Here, a technique of using a CO₂ laser to drive targets towards the laser spot via mass transport without requiring any device fabrication processes or special reagents was introduced. To prove the concept, single-stranded DNA (ssDNA) has been enriched by more than 100,000-fold in less than 4 minutes. The temperature and evaporation rate profile at the enriched area are measured alongside theoretical analyses and modeling to monitor and understand the physical process. The formation of aggregates

comprised of streptavidin Q-dots and biotin labeled exosomes with this method was demonstrated to show the capability of biosample detection, purification, and quantification. The method is not only simple and highly efficient, but also applicable to all types of biomolecules and bioparticles. Thereby promising a simple, cost effective and efficient solution for biological sample preparation for sensing, analytics, and diagnostics.

Chapter 1 Introduction

1.1 Single Cell Analysis

The fundamental unit of structure and function for all living organisms is the cell. All of life, whether single or multicellular, arose from pre-existing cells¹. The central challenge of biology is therefore to understand how individual cells process information and respond to perturbations. However, much of our knowledge is based on ensemble measurements where cell-to-cell differences are always present to some degree in any cell population². Before we can think about any cell analysis or study, we need to know what degree of heterogeneity there may be *in vivo* ³. The first form of heterogeneity arises from cell morphology. As shown in **Figure 1 (a) - (c)**, blood is composed of smooth, concave shaped, erythroid cells intermixed with irregularly shaped leucocytes and platelet cells. Neural tissue would reveal a network of glial cells and neurons, with long projections that interconnect to facilitate the propagation of signals while heart tissue would reveal stacks of filamentous cardiomyocyte cells. Indeed, phenotypic differences among cells are always present at a fine-enough resolution of inspection⁴. However, to effectively study and understand biological process and disease formation or progression, the second level -- intracellular heterogeneity (shown in **Figure 1 (d)**) must be accounted for. This can be difficult to achieve by standard biological techniques because analysis at the tissue or cell population level often result in the loss of cellular resolution and context. Furthermore, the source material may be dominated by a large number of cells that do not express a biomolecule of interest, resulting in a dilution of the signal below the lower detection limit of a technique. Shown in **Figure 2 (a)**, a lowly expressed biomolecule (red triangles) fails to be detected owing to the predominance of other species (white squares) in the aggregate sample. On the other hand,

cell populations can average out individual cellular coexpression patterns as shown in **Figure 2 (b)**. This makes it difficult to determine the biologically relevant ratios of biomolecular species that are inaccurately represented in the aggregate sample.

A standard technique to isolate single cell from a cell population is fluorescence-activated cell sorting (FACS), otherwise known as a flow cytometer. A state-of-the-art flow cytometer can screen millions of cells per second and sort out a specific, pure subpopulation out of heterogeneous mixtures for further biochemical analysis and genomic studies, thus enabling studies of rare events such as the isolation of stem cells, circulating tumor cells or many more. Single cells sorted by flow cytometer can be directly fed to a sequencing process. The recent implementation of advanced analysis technologies like next-generation sequencing has boosted our knowledge and understanding of the complex heterogeneity in cellular genomics epigenomics, and transcriptomics.⁵⁻⁷ However, the analysis of individual cell secretion is essential to fully understand the functional heterogeneity and decipher the underlying mechanisms of cellular interactions and communication. Intercellular signaling and formation of cellular networks is a highly dynamic process mediated by the expression and secretion of small proteins (e.g., cytokines) and other molecules which govern particular cell activities, behaviors, and functions. The sensitive monitoring and analysis of cell secretion provide invaluable information of the specific functionality and real-time activity of individual cells in either physiological or pathological process. However, such dynamic and accurate analysis is still an unresolved challenge.

The past few years have seen the advances in innovative strategies for both cell isolation and protein secretion analysis to enable single-cell resolution.⁸ For example, a large-scale micro engraved array which is able to generate the populational secretion profiles from single cells.^{9, 10}

Immunofluorescence staining is also widely used for protein study, yet the necessity of intensive washing steps dramatically impairs the temporal resolution of the analysis¹¹. Droplet microfluidics has been presented as a promising platform for single - cell sorting and analysis¹². However, subsequent cytokine quantification requires external readout means, such as flow cytometry or spectroscopic methods that increase the overall system complexity and only provide an end-point result. And while examples of optical sensors relying on surface plasmon resonance have also been developed^{8, 13–16}, these devices are still unable to associate the properties of exosomes directly with their cell sources up to the resolution level of single cells.

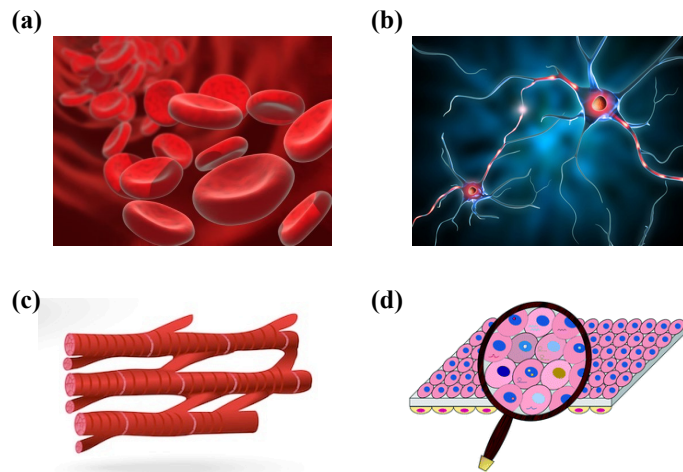


Figure 1.1 Cell heterogeneity. (a) - (c): cell type heterogeneity; (d) intra-cell heterogeneity

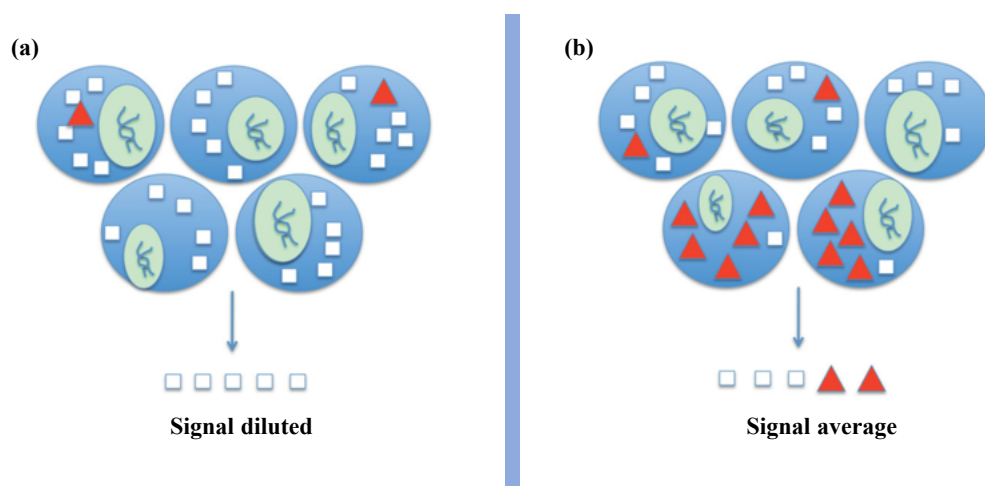


Figure 1.2 Loss of single-cell resolution is endemic to tissue-level analyses. The nucleus of the cell is depicted in the green oval situated in each cell. (a) Signal dilution; (b) Signal average⁴

1.2 Biosample enrichment

The main challenge in single cell secretion analysis is the limit-of-detection (LOD) and sensitivity.¹⁷ Thus, sample enrichment is an important step in the work flow of biosensing for disease detection and numerous biological or clinical processes. Most current techniques require specially designed devices tailored to specific chemical or physical characteristics of the target objects to enrich or capture them from the sample, which may increase cost and limit the enrichment factor.

The performance of sensors for organic and inorganic chemical compounds, DNAs, proteins, virus, bacteria, etc. are often measured by the minimum concentration of the target particles the sensors can detect. Hence, sample enrichment in solution phase is usually a necessary step in the work flow of sample processing. Today's sample enrichment methods are mostly material specific, utilizing the unique physical, chemical, and biological properties of the target particles. The methods usually involve a sample capturing processing, followed by sample release. For example, the target particles are captured by flowing samples through a column of

high surface area while having a strong affinity for the target particles. After target capture, particles are then released into the elution buffer^{18–22}. Such processes of sample enrichment and extraction require multiple steps and have limited throughputs.

Besides using chemical properties of binding affinity, there have been numerous approaches using physical properties of particles for enrichment. These approaches use passive or active devices based on the mechanisms that control the motions of particles in the sample solution. Passive designs can use Van der Waals attraction²³, Brownian motion²⁴ and charged dipole interaction²⁵. Active designs make use of hydrodynamic force^{26–29}, dielectrophoretic (DEP) effect^{30–32}, electrophoretic (EP) effect^{33, 34}, magnetophoretic effect^{35, 36}, thermophoretic^{37–41} and IR induced differential evaporation⁴². The DEP device and operation conditions (e.g. electric signal frequencies) need to be adjusted to work for certain particles based on their Clausius-Mossotti (CM) factor, which determines whether the particles move towards or away from the electric field gradient. The device design and operation condition also critically depend on the particle volume and the ionic strength of buffer. Hence DEP devices are more effective for larger particles such as bacteria and mammalian cells^{43, 44}. In contrast, devices utilizing the electrophoretic (EP) effect work more effectively with smaller particles such as proteins and nucleic acids. While the EP effect has been widely established with gel electrophoresis to separate charged biomolecules, the technique is often used for molecular analyses such as western blot (for proteins) and southern blot (for DNAs) instead of sample enrichment for applications such as in-vitro diagnosis (IVD) and point-of-care. Thermophoresis can be used to trap DNAs in a microscopic volume through convection induced by temperature gradient⁴⁰. Since particles tend to migrate from hot areas to cold areas against the thermal gradient, the thermophoretic effect generally causes depletion rather than accumulation of particles near the

heat source. However, by adjusting the chamber thickness to tailor the thermal gradient across the chamber and controlling the salt concentration, particle enrichment can occur near the hot spot. Using this technique, SYBR-stained DNA in the solution is enriched from an initial concentration of 0.5 nM to uM concentration range in 3 minutes, giving rise to an enrichment factor of over 1000. Figure 1.3 lists some current technology for biosensing technology for cell secretion enrichment.

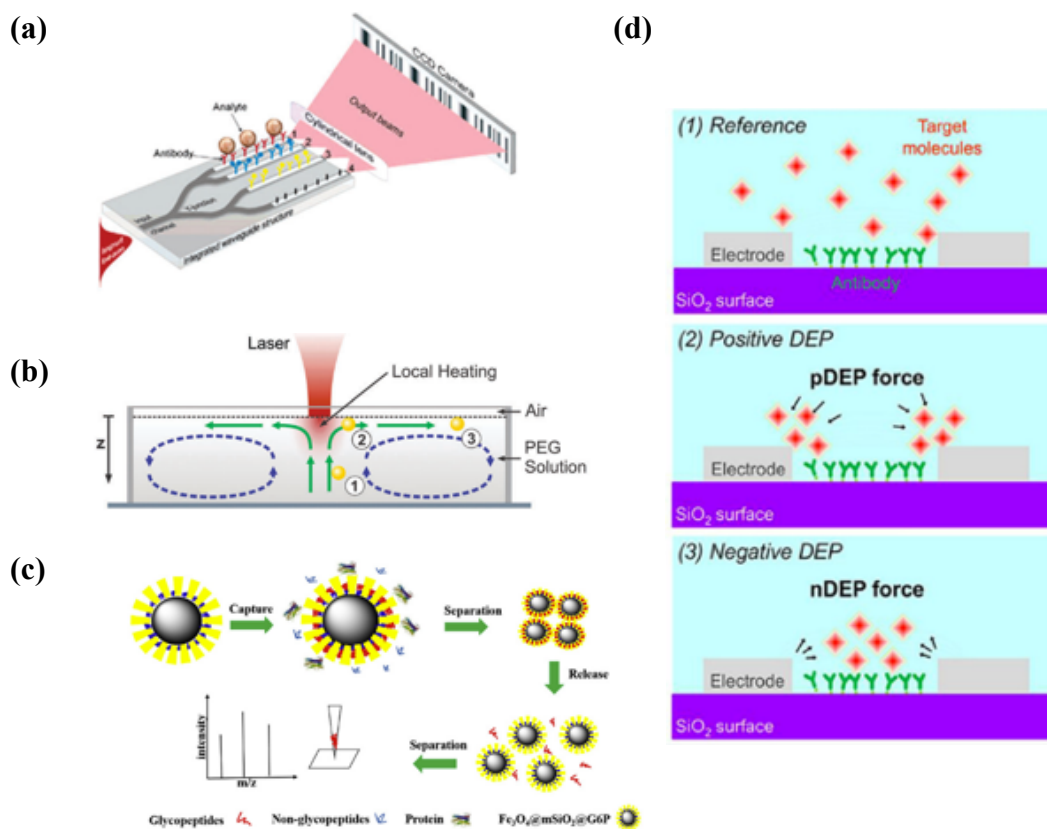


Figure 1.3 Current technology for cell secretion enrichment. (a) Microfluidic device⁴⁵; (b) Thermophoretic⁴⁶; (c) Chemical properties of binding affinity²⁰; (d) Schematic of positive and negative DEP force acting on bio macromolecules at the presence of a non-uniform electric field¹⁶.

1.3 Dissertation outline

The dissertation presented a single-cell assay and a enrichment method with the following salient features: (a) achieve high throughput long term single cell culture and quantitative study; (b) Quantitative analysis of single cell secretion non-invasively; (c) Capability to relate single cell secretions to genomic analysis; (d) Enrich biosamples for downstream analysis

Chapter 2 features fabrication and characterization of high through put single cell culture chip.

Chapter 3 will demonstrate how to use the single-cell assay to quantify single sell secretions and gene expression.

Chapter 4 will introduce an IR-induced biosample enrichment method. The enrichment capabilities and application in the analysis of single cells will be demonstrated.

Chapter 2 Fabrication of the single-cell assay to achieve the high throughput long term single cell culture and quantitative study

2.1 Overview

In this chapter, I will present the design of novel chips which can be used for single cell loading and culture. (1) PDMS micro through holes arrays using PDMS lift-off process, (2) Binding the mesh with filters to collect the cell secretion from the bottom. (3) Demonstrate the single MCF7 cells loading and time-lapse observation

2.2 PDMS micro through holes arrays using PDMS lift-off process

The PDMS single cell loading mesh was made via the PDMS lift-off (peel-off) process. A similar lift-off process had been reported by Park et al.⁴⁷ and Guo et al.⁴⁸; however, these methods require precise process control and sophisticated skills. The fabrication process we developed here utilizes a simpler peel-off process with high tolerance and a large process parameter window.

The process flow is summarized in **Figure 2.1**. To define the mesh pattern, 6 μm thick NR9-3000PY negative photoresist (Futurrex, Frankling, NJ, USA) was spin-coated on a 4-inch Si wafer at 800 rpm for 40 seconds. The wafer was soft baked at 150°C for 1 minute, followed by 90 seconds of UV exposure (Karl Suss MA6 Mask Aligner) and 100°C post-exposure baking for 1 minute. The patterns were revealed after resist development. A two-dimensional array of

100 μm deep, 40 μm diameter patterns were then etched by deep reactive ion etching (DRIE) process (Oxford Plasmalab 100) using the photoresist as an etch mask. In the DRIE process, SF_6 gas was flowed at 100 sccm for 11 s during the etching cycle, followed by a passivation cycle with C_4F_8 gas flowed at 80 sccm for 7 s. The etching rate was $\sim 0.65 \mu\text{m}$ to $\sim 0.7 \mu\text{m}$ per cycle and took about 150 cycles to etch $\sim 100 \mu\text{m}$ Si. The passivation of the Si wafer surface process was added before spin-coating the PDMS. 2 drops of 3-Aminopropyltriethoxy-silane, (Aldrich, 0762888) and the Si wafer were put in a vacuum desiccator for 30 mins. This process is to aid the release of PDMS and prevents the PDMS from adhering to the Si wafer. After passivation, $\sim 50 \mu\text{m}$ thick uncured PDMS (Sylgard 184, Dow Corning, MI) with a premixed 1:1 ratio (v/v) with hexane was spin-coated on the Si wafer at 1500 rpm for 60 seconds. The PDMS coated wafer was baked in a 65°C oven for 90 minutes. A 2 mm thick PDMS ring with 12 mm inner diameter and 14 mm outer diameter was attached to the Si wafer by using uncured PDMS to define the cell loading area. The whole assembly, with the patterned wafer and the PDMS ring, was cured in a 65°C oven for 90 minutes. The PDMS ring helps enhance the mechanical strength and flatness of PDMS mesh to be formed by photoresist lift-off. To produce the mesh with a 2D array of through holes, the wafer was immersed in acetone and sonicated for 5 minutes twice, and then was rinsed with methanol and isopropanol and dried by nitrogen gas. The acetone sonication process allowed the solvent to diffuse through the hexane-mixed PDMS layer to reach the NR9-3000PY photoresist, causing the resist to swell and lift off the PDMS layer atop the NR9-3000PY photoresist. After baking the wafer in a 75°C oven for 5 minutes to ensure solvent evaporation, one could easily separate the PDMS mesh with an array of through holes from the Si wafer by holding the PDMS ring.

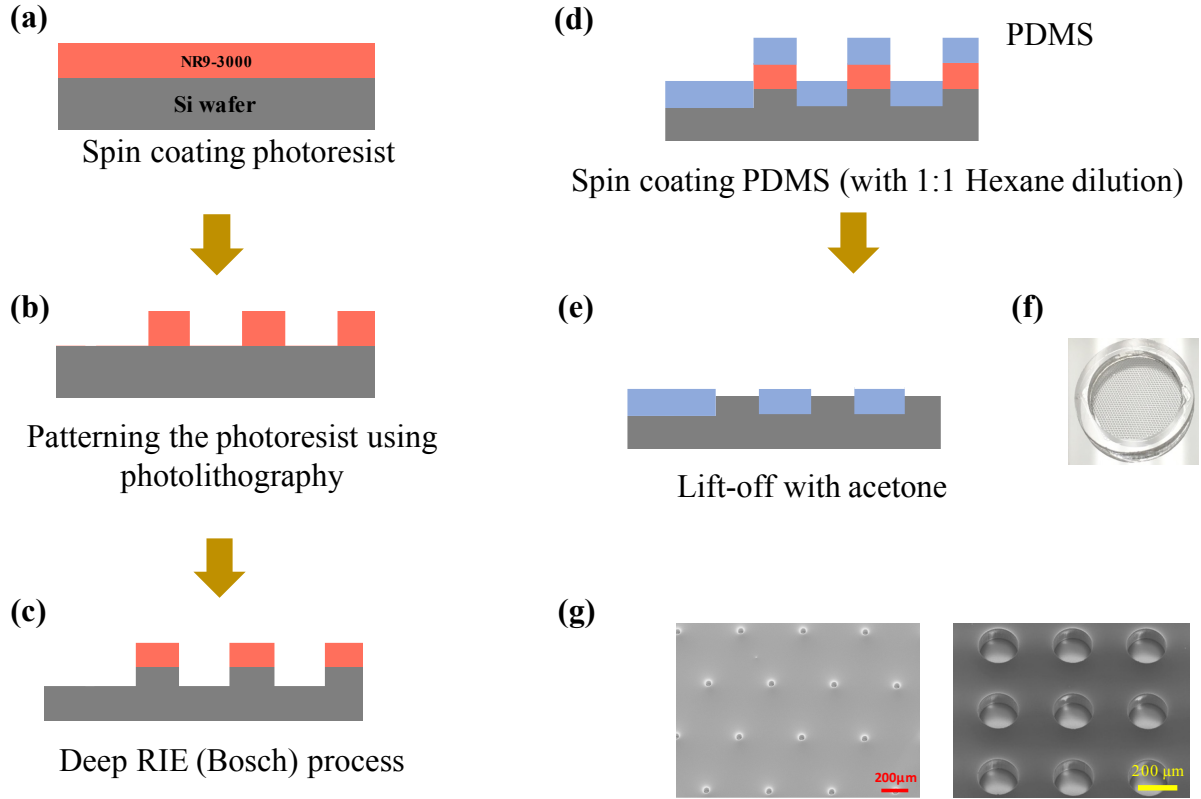


Figure 2.1: Process flow for fabrication of PDMS mesh to aid cell loading. a) Prepare a clean Si-wafer and spin-coat 6 μm photoresist NR9-3000 PY on top. b) Pattern the photoresist by photolithography. c) Using photoresist as mask for deep RIE process to form 100 μm deep, 40 μm diameter mesh. d) Spin coat PDMS with 1:1 hexane dilution e) Lift-off the PDMS atop the photoresist with acetone to form PDMS through-holes. f) Photograph of the PDMS mesh supported by a PDMS ring. g) SEM images of the PDMS mesh with a 2D array of through holes to aid cell loading. Different size and aspect ratio can be easily achieved.

2.3 Single cells loading and culturing

The PDMS mesh was adhered to a cell culture dish to form microwells to help load and guide the positions of single cells, and then removed at user's choice, to allow single cell culturing without space restrictions.

The PDMS mesh was treated with oxygen plasma (100 Watl for 30 seconds) to provide enough bonding strength with the culture dish to sustain cell loading, but also allow ease in

separating the mesh by mechanical shear after cell loading. After filling the wells with the culture medium, the cell suspended medium was added and the device was centrifuged at 140-g for one minute to drive the cells into the wells. The cell-laden sample was then incubated at 37°C for 4 - 6 hours until cells were attached to the bottom of the culture dish. The PDMS mesh can then be gently removed from the glass substrate without disturbing the cells. The process flow is illustrated in **Figure 2.2**.

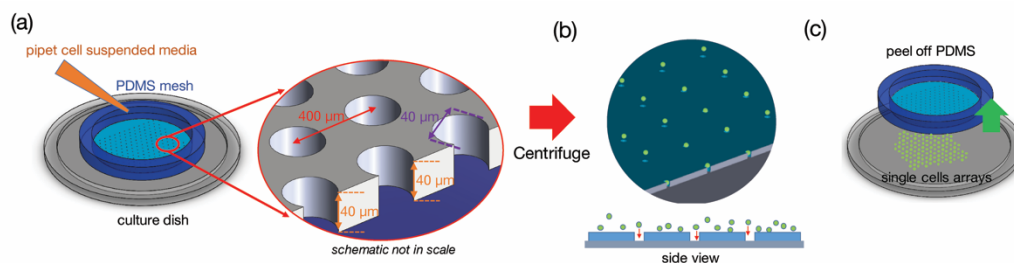


Figure 2.2: Single-cell loading process. a) Loading single cells onto a culture plate utilizing a PDMS mesh which can be removed afterward. b) centrifuged at 140-g for one minute to drive the cells into the wells. c) The mesh was removed after cell attachment.

Figure 2.3 (a) shows the image of GFP transfected MCF7 cells under a 4X objective lens (BE-II 9000, Keyence) after 6 hours after the centrifuge-assisted cell loading. About 70% of the positions defined by the PDMS mesh contained single cells, and less than 10% of positions contained two or more cells. Cell loading efficiency and cell number at each position depend on the size of mesh, packing density of mesh and cell density. The data presented here uses a PDMS mesh with 40 μm diameter holes with center-to-center spacing of 400 μm under a cell density of 160 cells/μL. These parameters have resulted in good single cell yield and density to support most single-cell studies. Since the cells are under natural culture conditions, the long-term culture and time-lapse observation can easily be achieved. **Figure 2.3 (c)** shows single cells that were cultured for up to 96 hours in time lapsed studies.

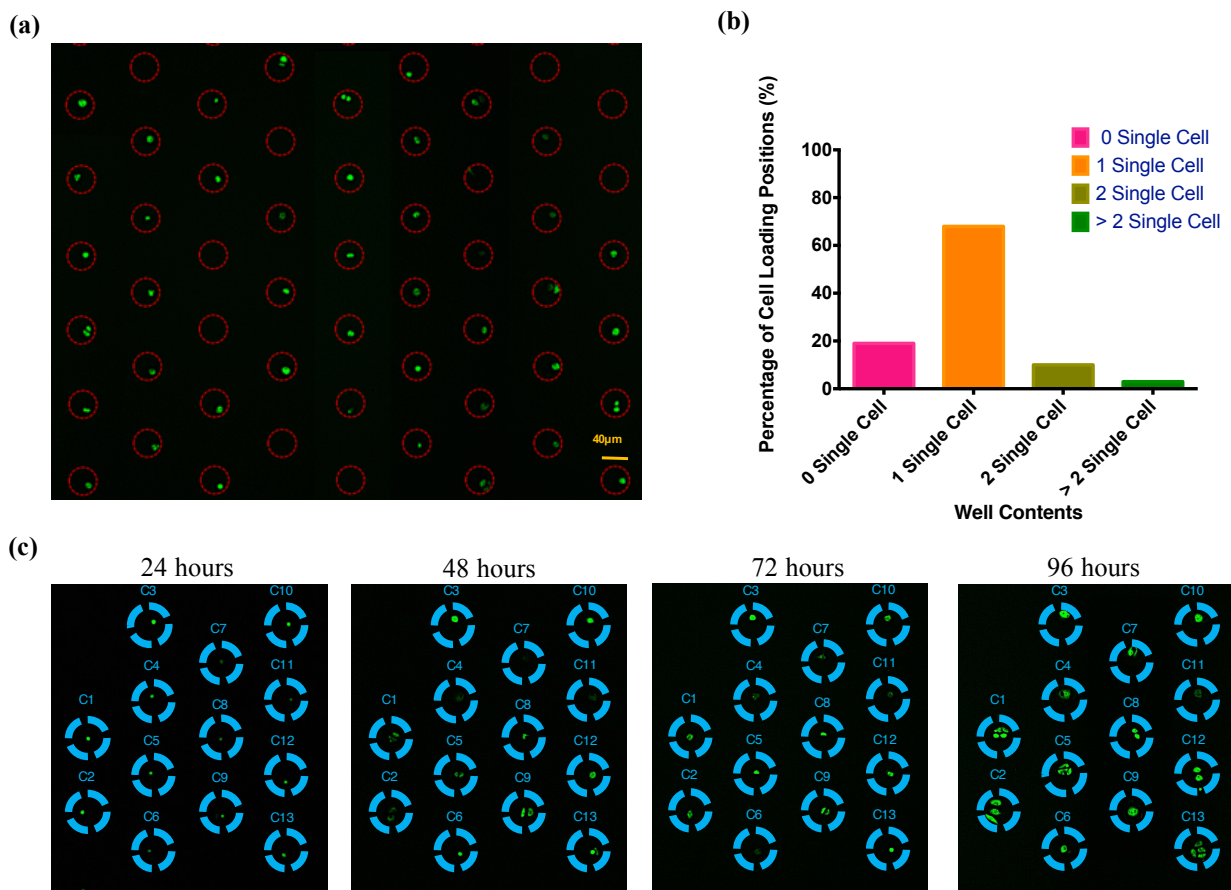


Figure 2.3 Cell Loading and culturing. (a) Distribution of GFP transfected MCF7 cells. (b) Statistic distribution of cell numbers. (c) Time-lapsed observations of single cell culture. All cells survived and proliferated.

2.4 Combining PDMS arrays to filters

Another way to use the PDMS mesh is to bind it with a filter. Here I choose the hydrophilic polyester track etch (PETE) membrane filters (Sterlitech) with 0.8 μm pores to allow cell seeding and collection of cell secretions through the membrane pores into the underlying microwells at the same time. The fabrication process was summarized in **Figure 2.4**. To bond the PDMS through-holes mesh onto the PETE membrane filter, the PETE membrane was activated in an oxygen plasma chamber for 7 min (600 mTorr, 100 W) and then immersed in a 5% water

diluted 3-Aminopropyltriethoxy-silane, (APTES) (Aldrich, 0762888) solution on a 80 °C hot plate for 20 minutes. The APTES solution was covered during the heating process to prevent water evaporation. Afterwards the APTES-treated PETE membrane was bonded to the oxygen plasma activated (600 mTorr, 100 W) PDMS film with through-holes. When the PETE membrane was brought to contact with the PDMS through-hole film, an irreversible bond was formed almost immediately. After the bonded sample was dried in air for 24 hours, the chip was ready for single cell placement and culturing. After cell loading, the device can be placed in a culture dish or on a 1536 wells plate (micro well plates) to collect secretions from bottom.

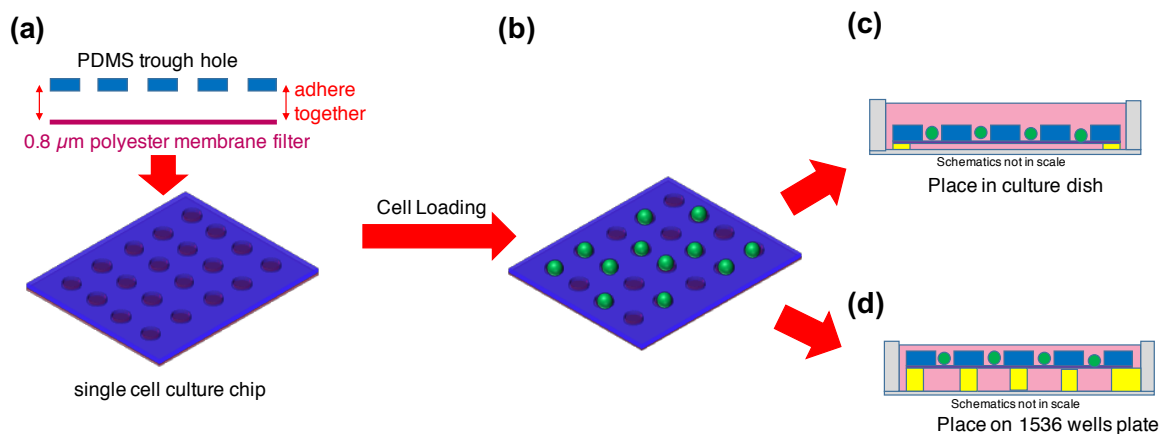


Figure 2.4: Fabrication process of filter binding to PDMS mesh (a) Binding PDMS mesh with filter (b) to achieve suspension cell loading (c) and secretion collection from both top and bottom.

2.5 Summary

In this chapter, I presented the fabrication of PDMS micro through holes arrays using PDMS lift-off process. This PDMS mesh was adhered to a cell culture dish to form microwells to help to load and guide the positions of single cells, and then removed, at user's choice, to allow single cell culturing without space restrictions. MCF7 single cells loading and long-term

culturing was presented, showing that the single cell loading yield is about 75%. The technology and process can also maintain cell viability at single cell levels with the closest neighboring cells being 400 μm apart. This PDMS mesh can also bind with the filter to achieve the cell secretion collection from the bottom. that allows for harvest of single-cell secretions noninvasively.

This chapter is based on the following papers: Yu-Jui Chiu, Wei Cai, Yu-Ru V. Shih, Ian Lian, and Yu-Hwa Lo. A single-cell assay for time lapse studies of exosome secretion and cell behaviors, *Small*. 12 (2016) 3658-3666 and Wei Cai, Yu-Jui Chiu, Valya Ramakrishnan, Yi-Huan Tsai, Clark Chen, Yu-Hwa Lo, A single-cell translocation and secretion assay (TransSeA), *Lab on a Chip*, 18 (20): 3154-3162, 2018. The dissertation author was the primary investigator and author of this paper.

Chapter 3 A Single Cell Assay for Quantifying Single Cell Secretions and Gene Expression

3.1 Overview

In the last chapter, a single-cell assay was presented. This technology is capable to culture single cells of different cell types in a nature culture environment. This feature is essential for downstream analysis of secretion from single cells. To demonstrate the unique features as a single-cell assay, I will quantify the rate of exosome secretion by single cells over a period of 24 - 96 hours under different perturbation and also relate single cell secretions to genomic analysis.

3.2 Workflow from Cell loading, translocation to Exosome Collection

The overall work flow of the single cell assay is shown in **Figure 3.1. Figure 3.1 (a) - (c)** which summarizes the single cells loading process described in chapter 2. The bioprinting method was combined with the single-cell assay to collect secretions from single cells regularly without disturbing.

In order to collect the exosomes, a surface functionalized collection glass slide was placed 0.1 mm above the cells. The process design for the collection of single cell secretions is

illustrated in **Figure 3.1 (d) - (f)**. A 2 mm thick Computer Numerical Control (CNC) machined acrylic fixture was placed on the cell culture glass substrate to define the 0.1mm space between the single cell array and the surface-treated glass slide which collects exosomes secreted by single cells. The collection glass has 30 μm wide fiducial markers that are visible under low power microscopes and cellphone cameras. These fiducial markers served as the references to register the cell sources that produced exosomes captured by the glass slide at designated locations. In the experiment each anti-body treated glass slide captured exosomes secreted by each batch of single cells at certain time point, and those captured exosomes were subsequently labeled with another biotinylated antibody, which was subsequently bonded to streptavidin modified Quantum Dots (Q-dots) (Life Technologies), as shown in **Figure 3.1 (f)**. Using an inverted fluorescent microscope to count Q-dots over each area corresponding to the location of the single cells, one can investigate the properties of exosomes produced by the single cell source. To prove the concept of the single-cell assay, proteins CD63, CD9, and CD81 on the surface of exosomes was investigated in this section. As a platform technology, the method can be used to collect any single cell secretions with specifically treated collection glass slides.

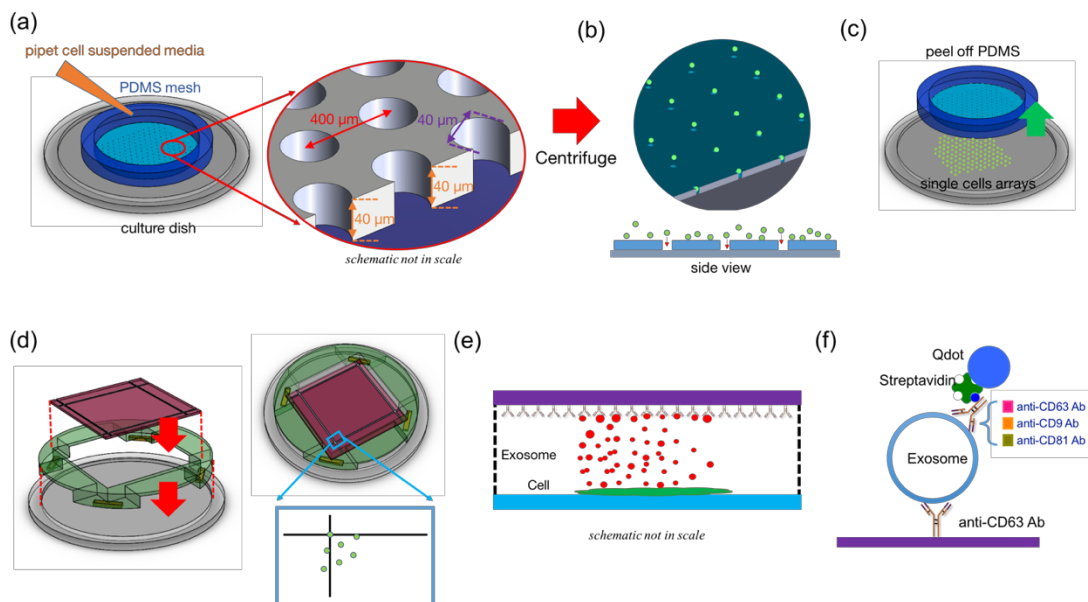


Figure 3.1 Single-cell assay used for analyzing exosome secretion. (a) and (b) Loading single cells onto a culture plate utilizing a PDMS mesh which can be removed afterward. (c) The mesh was removed after cell attachment. (d) and (e) A surface functionalized glass slide was placed on the support frame 100 μm above the cells, the glass slide collected exosomes secreted by the corresponding cells. The fiducials on the cover glass served as registration marks. (f) The captured exosomes were labeled with another biotinylated antibody and streptavidin-conjugated Quantum dots to become visible under fluorescent microscope.

3.3 Cell culture

GFP-transfected MCF7 (MCF7/GFP) cells and GFP-transfected MDA-MB-231 (MDA-MB-231/GFP) cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (Pen/Strep) in a humidified incubator at 37 °C with 5% CO₂. Cells were harvested using 0.05% (w/v) trypsin EDTA when 80% confluence was attained. 3X culture media were added to neutralized trypsin before centrifuging down the cells. On the other hand, MCF10A cells were cultured in DMEM/F12 medium supplemented with 5% (v/v) horse serum, 20 ng/ml EGF, 0.5

µg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, and 1% (v/v) penicillin/streptomycin (Pen/Strep) in the same incubator described above.

MCF10A cells were harvested using 0.05% (w/v) trypsin EDTA when 80% confluence was attained. Once cells were dislodged, 4.0 ml of resuspension medium made with DMEM/F12 medium supplemented with 20% (v/v) horse serum and 1% (v/v) Pen/Strep was applied to the plate and pipette to break up cell clumps before centrifuging with 150g for 3 minutes. For each cell line, the cell concentration was measured by a flow cytometer (Accuri C6) before dilution with suitable culture media.

CMK3 cells were cultured in DMEM/F12 medium supplemented 2% (v/v) B27 supplement, 1% (v/v) penicillin/streptomycin (Pen/Strep), 1% (v/v) Glutamax, 10 µg/ml EGF, 10 µg/ml FGF and 1.2mL heparin in a humidified incubator at 37 °C with 5% CO₂.

3.4 Antibody immobilization on cover slide

To immobilize anti-CD63 antibodies on the cover slide for exosome capture, the cover slide was diced and silanized in 4% v/v (3-mercaptopropyl) trimethoxysilane (MPS) for 30mins with 200 rpm spin speed at room temperature. The silanized cover slide was then washed three times with ethanol, followed by 100°C heating for 30 mins until it fully dried. The cover slide was incubated in 0.1 mM cross-linker, Sulfo-GMBS, dissolved in phosphate buffered saline (PBS, pH 7.4, Gibco) for 40 minutes at room temperature, and then washed by PBS for 3 times. The cover slide was then incubated in 0.05 µM anti-CD63 Ab (Ancell) at 4 °C for 2 hours, followed by PBS wash for 3 times. The cover slide was then immersed in PBS with 5% (w/v) bovine serum albumin (BSA) at 4°C for 30 minutes to passivate non-reacted Sulfo-GMBS. After PBS wash for 3 times, the functionalized cover slide was stored in PBS at 4°C and ready to use.

3.5 Imaging and Counting

After exosomes were collected to the functionalized cover slide, the cover slide with exosomes was fixed with 4% (v/v) paraformaldehyde in PBS at room temperature for 30 minutes. The slide was then washed 3 times with PBS, incubated in 0.05uM biotinylated antibodies (Ancell Inc.) solution at 4 °C for 2 hours, and washed with PBS for 3 times again. In this demonstration, three types of biotinylated antibodies (anti-CD63 Ab, anti-CD9 Ab, or anti-CD81 Ab) might be used, depending on which surface protein was to be characterized. The cover slide was subsequently incubated in 10 nM streptavidin-coated Q-dots solution at room temperature for 1 hour, followed by three times of 50°C mixture of 1X tris-buffered saline (TBS) and Polysorbate 20 (TBST) wash and 2 times of 50°C deionized (DI) water wash before dehydrated in 25%, 50%, 75%, 90%, 100%, 100% ethanol, respectively. The sample was then imaged by using an inverted fluorescent microscope (BE-II 9000, Keyence) with excitation/emission filters of 405/10 nm and 536/40 nm, respectively. The fluorescent images were processed through haze reduction and black balance algorithms, and Q-dots were counted by using an object counter module in the microscope software (BZ-II Analyzer).

3.6 Characterization of the platform

The maximum exosome capturing capacity of the glass slide, immobilized with anti-CD63 Ab, was tested with samples containing a high concentration ($\sim 2 \times 10^4/\mu\text{L}$) of exosomes in phosphate buffered saline (PBS) solution. It was determined that within the $110 \mu\text{m} \times 150 \mu\text{m}$ microscopic field of view under 100X objective lens, a maximum number of ~ 1500 anti-CD63 Ab conjugated Q-dots can be bonded to the surface, setting the upper limit of detectable density

of exosomes. Therefore, the exosome collection time in this study was limited to 3 hours so that the highest number of Q-dots conjugated with exosomes was around 500, which is well within the detection limit. To characterize the spatial resolution of the method and the crosstalk from capture of exosomes secreted by neighboring cells, exosome counts over areas without corresponding cells but with cells in neighboring areas were analyzed. As shown in **Figure 3.2**, the exosome counts in those cell-absent areas were comparable with the counts in the background level due to non-specific binding of Q-dots, about 25 over the field of view. The results confirmed that about 3% of signal might come from crosstalk caused by exosome diffusion.

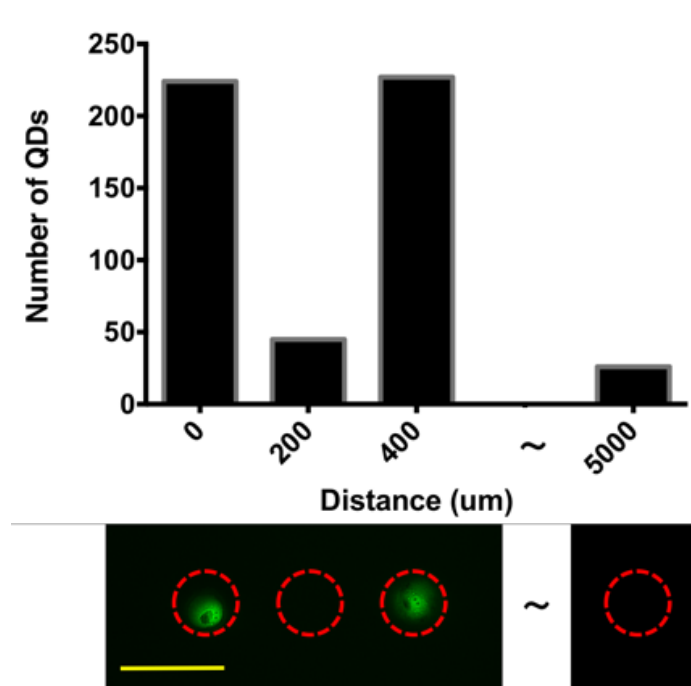


Figure 3.2 Evaluation of crosstalk due to exosomes secreted by the neighboring sites. The result shows that the crosstalk is slightly above the background level and contributes to about 3% of the signal. The scale bar in the lower picture represents 200 μm .

3.7 Single cell exosome secretion rate

Three breast cell lines, MCF10A, MCF7, and MDA-MB-231, were used to measure the exosome secretion rate from each single cell. The exosome secretion rate by each cell type was obtained by measuring the secretion rate from 8 to 10 individual cells of each type. The number of exosomes for each cell was counted over a field of view of 110 μm x 150 μm , an area that was verified to be large enough to include all exosomes secreted by the single cell from the corresponding position. The collection time was chosen to be 3 hours for each measurement with each experiment typically lasting for a period of 96 hours. During the measurement period, single cells may divide at certain points of time. When the cell number increased due to cell division, the measured number of exosomes was normalized to the cell number to obtain the single cell exosome secretion rate. As shown in **Figure 3.3 (a)**, each MCF7 cell and MDA-MB-231 cell had a similar exosome secretion rate of 60 - 65 exosomes per hour. However, each MCF10A cell secreted about 2.8x more exosomes than each MCF7 or MDA-MB-231 cell. **Figure 3.3 (b)** shows that exosomes produced by MCF10A cells and captured by the anti-CD63 Ab probes, only ~ 31% of them contained CD9 and almost none of them contained CD81. On the contrary, among the CD63 positive exosomes produced by MCF7 cells, as many as 89% contained CD9 and about 20% contained CD81. For those CD63 positive exosomes produced by MDA-MB-231 cells, 93% to 97% of them were CD9 and CD81 positive. Our results support the previous hypothesis with quantitative data unavailable before that tetraspanins CD9 and CD81 may be related to malignancy of cancer cells^{49, 50}.

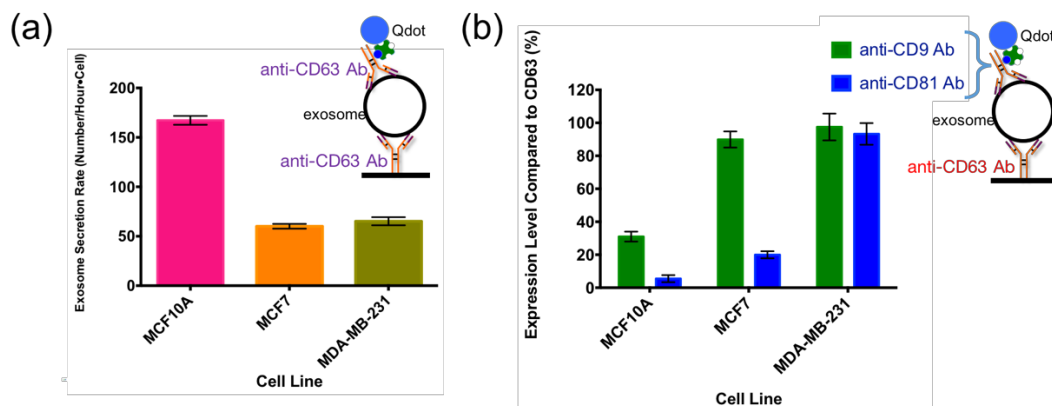


Figure 3.3 Expression level of different surface marker on different cell lines. (a) Exosome secretion rate (in number/hour-cell) characterized by CD63 protein labeled with biotinylated anti-CD63 antibody and Q-dots. (b) The percentage of CD9 and CD81 positive exosomes among all the exosomes captured by anti-CD63 antibody.

3.8 Time-lapse observation of exosome secretion rate under various conditions

As an open platform technology, the single-cell assay can be applied in various culture conditions.

It has been reported that microtubule-targeting drugs, e.g. paclitaxel, could reduce exosome release under non-cytotoxic doses⁵¹; however there has been no quantitative study on the magnitude of reduction. For a quantitative study to resolve the effect from each single cell, both MCF7 and MDA-MB-231 cell lines were investigated using the aforementioned method. The cells were cultured for 48 hours before applying the drug. The paclitaxel drug was dissolved in PBS and mixed with exosome-free culture medium to achieve a final concentration of 5 ng/mL. After adding the drug containing medium into the culture dish, CD63 positive exosomes were collected for 3 hours using the anti-CD63 Ab immobilized cover slides. After exosome collection, the cells were imaged and the Q-dots labelled exosomes at the corresponding

positions to the cells were imaged. Every 24 hours from the last collection, 2 mL of new exosome-free medium with 5 ng/mL paclitaxel would be replaced. The above steps were repeated throughout the 96 hours of experiment process.

Figure 3.4 shows the effects of paclitaxel on the exosome secretion rate for MCF7 and MDA-MB-231 cells. We observed a sharp decrease in the secretion rate of CD63 positive exosomes for MCF7 cells after 24 and 48 hours of treatment. On the contrary, MDA-MB-231 did not respond to paclitaxel in the first three hours, and the exosome secretion rate decreased by only $\sim 20\%$ after 24 hours. These results provide more quantitative information to support the notion that MDA-MB-231 may have higher chemo resistance than MCF7⁵² as MDA-MB-231 was less responsive to paclitaxel. To further quantify the effect of the drug on the expression levels of CD9 and CD81 among the CD63 positive exosomes, we define the impact factor as **Equation (3.1)**.

$$F_{CD9} = \left(\frac{R_{CD9}^*}{R_{CD63}^*} - \frac{R_{CD9}}{R_{CD63}} \right) / \left(\frac{R_{CD9}}{R_{CD63}} \right) \text{ and } F_{CD81} = \left(\frac{R_{CD81}^*}{R_{CD63}^*} - \frac{R_{CD81}}{R_{CD63}} \right) / \left(\frac{R_{CD81}}{R_{CD63}} \right) \quad (3.1)$$

where R and R^* represent the secretion rate under unperturbed and perturbed (by drugs, pH value, etc.) conditions. $F = 0$ means the perturbation has no effect while $F > 0$ and $F < 0$ means the perturbation increases and decreases the expression level of specific protein in exosomes respectively. The inserts in **Figure 3.4** show the impact factor of the drug-induced perturbation on MCF7 and MDA-MB-231 cells. The data show that, among all the CD63 positive exosomes, the drug has positively impacted the expression level of CD81 for MCF7 cells but negatively impacted the expression level of CD81 for MDA-MB-231 cells. On the

other hand, the drug has a modest effect on the expression level of CD9 for both MCF7 cells and MDA-MB-231 cells.

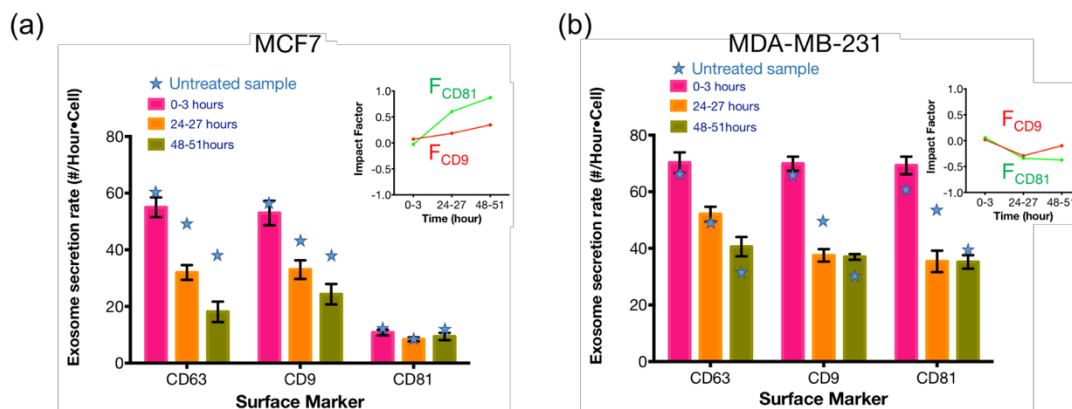


Figure 3.4 Expression level of different surface markers after paclitaxel treatments for (a) MCF7 and (b) MDA-MB-231. The star symbols indicate the secretion rate without paclitaxel treatment. The main figures show the exosome secretion rates (in number/hour-cell) for CD63 positive exosomes that are simultaneously CD9 or CD8 positive. The inserts show the impact factor (defined in text) of the drug on MCF7 and MDA-MB-231 cells regarding CD9 and CD81 expressions.

Studies have demonstrated how the exosome release from a single cell may become affected when the pH falls lower than the physiological value^{53, 54}. Here, a more quantitative study of exosome secretion in an acidic environment was conducted through this single cell platform. Similar to the paclitaxel test, the cells were cultured for 48 hours before adding 2 mL exosome-free culture media titrated with HCl to the final pH value of 6.7. Surface functionalized glass slides were then placed on the fixture to collect exosomes from single cells. After 3 hours of collection, the cells and the collected Q-dots labeled exosomes were imaged by the fluorescent microscope. The above procedures, including medium replacement, exosome collection, and microscope observations of cells and exosomes at the corresponding positions, were repeated every 24 hours.

The evaluation of exosome secretion and the impact factor due to the pH change was shown in **Figure 3.5**. The method is described before the in-drug treatment experiment. The secretion rate of CD63 positive exosomes for both MCF7 cells and MDA-MB-231 cells was not obviously affected in the acidic environment. However, the expression levels of CD9 and CD81 among the CD63 positive exosomes of MCF7 cells dropped significantly (i.e. impact factor ~ -0.5) within the first 3 hours under the acidic condition. Such drops appeared to be transient rather than a permanent effect since in 24 - 27 hours, the expression levels of CD9 and CD81 more or less recovered to previous values at physiological pH. The expression levels of CD9 and CD81 for MDA-MB-231 cells however, were not apparently affected by the pH value change. The results seem to suggest that both MCF7 and MDA-MB-231 cells are relatively resilient to the acidic environment in terms of their exosome secretion rate and expression level of surface proteins.

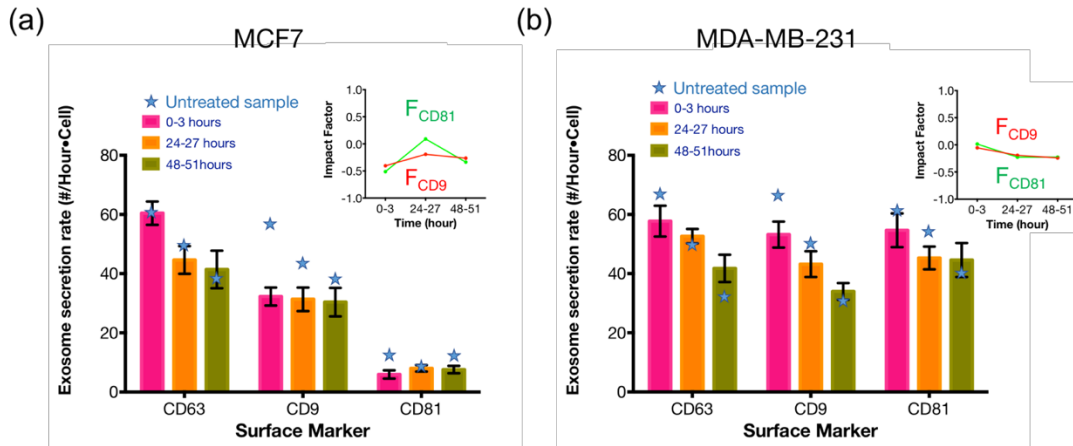


Figure 3.5 Expression levels of different surface markers after being cultured in lower pH (6.7) media for (a) MCF7 and (b) MDA-MB-231. The star symbols indicate the secretion rate in normal pH (7.4) medium. The main figures show the exosome secretion rates over time for CD63 positive exosomes that are simultaneously CD9 or CD8 positive. The inserts show the impact factor of the pH change on exosomal CD9 and CD81 expressions for MCF7 and MDA-MB-231 cells.

Besides drug and environmental (pH) stress, cells that are responsive to certain proteins such as transforming growth factor beta (TGF- β) is have also been investigated. TGF- β is a protein that was reported to induce epithelial-to-mesenchymal transition in MCF7 cells. In this experiment, 10 ng of TGF- β was added into 10 mL exosome-free medium to culture MCF7 single cells. Again, at every 24 - hour time interval and after each 3 - hour exosome collection and cell observation, the above medium was replaced and the processing steps were repeated. As shown in **Figure 3.6**, although the initial exosome secretion rate of MCF7 cells was around 70% of the rate without the growth factor TGF- β , the exosome secretion rate surged to 120% after 24 hours. The impact factor analysis indicates that after the TGF- β treatment for 48 hours, the expression level of CD81 among the CD63 positive exosomes rose significantly (with impact factor reaching +2) while the response of CD9 to TGF- β was modest.

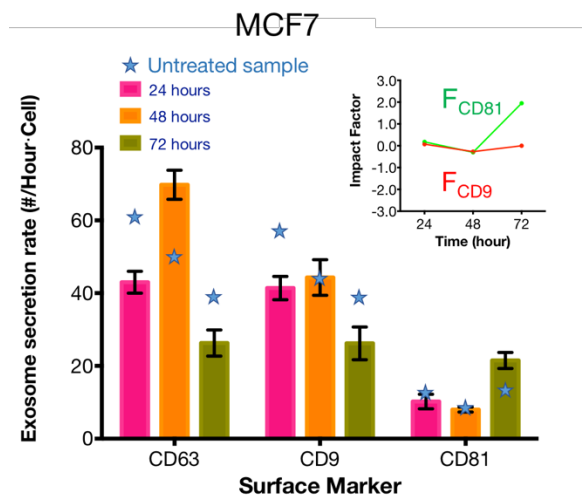


Figure 3.6: Expression level of different surface markers after the TGF- β treatment for MCF7. The star symbols indicate the secretion rate without TGF- β treatment. The main figure shows the exosome secretion rates over time for CD63 positive exosomes that are simultaneously CD9 and CD81 positive. The insert shows the impact factor of the perturbation on MCF7.

3.9 Relate single cell secretions to genomic analysis

In this section, single cell secretions and genomic analysis was demonstrated with this single cell assay. GFP-labelled CMK3 cells (a short-term passaged, patient derived glioblastoma line) were enumerated.

The workflow is summarized in **Figure 3.7**. After binding the PDMS mesh with the filter (detailed fabrication process was described in Chapter 2), a bioprinting method can be used to collect the single cells secretion from the top or achieve holistic secretion collection from the bottom.

Using the bioprinting method, CD63 positive exosome secreted from 193 single CMK3 cells are summarized in **Figure 3.8** where the exosome secretion rate is binned with an increment of 10. The data shows an extraordinarily large inhomogeneity in exosome secretion rate among individual cells, ranging from 2 exosomes to 218 exosomes in a 3-hour collection period. The variation is too large to be fitted by a single (Gaussian) distribution function. Instead the curve can be fitted by two distinct distributions, one with an average secretion rate around 40 exosomes per 3 hours and another with an average secretion rate of 115 exosomes per 3 hours.

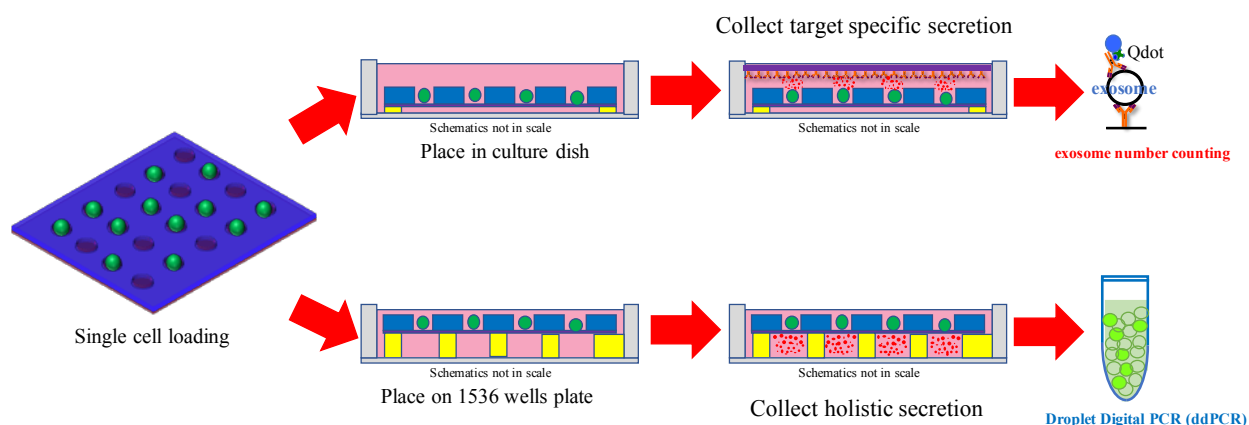


Figure 3.7 The single cell assay for cell secretion and genomic analysis

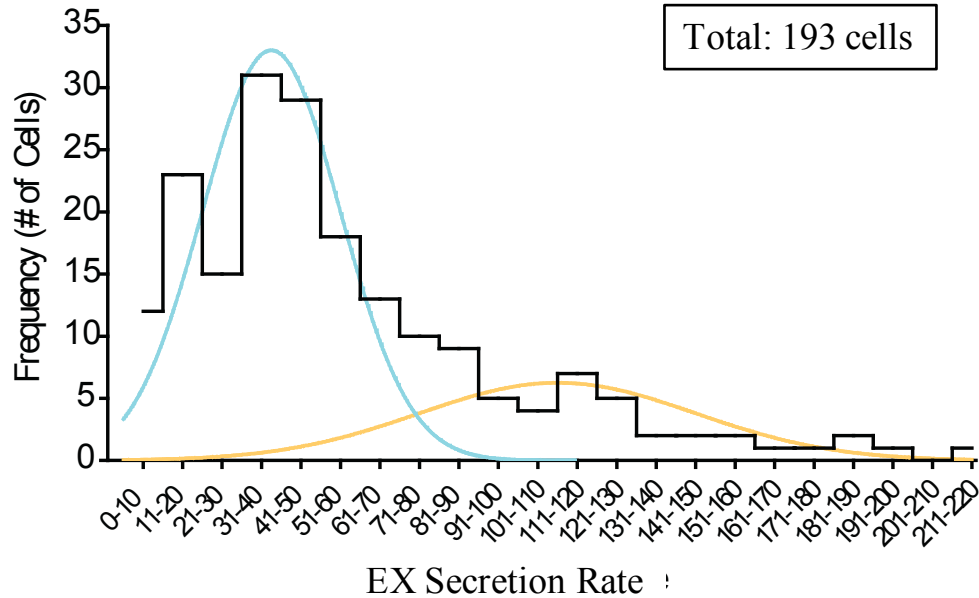


Figure 3.8 Summary of the exosome secretion rate from 193 single cells. The data show extraordinarily large inhomogeneity in the exosome secretion rate among individual cells and cannot be fitted by a single Gaussian or other typical distribution functions. They are best fitted by two distribution curves, one with an average secretion rate of around 40 exosomes per 3 hours (blue) and another with an average secretion rate of 115 exosomes per 3 hours (yellow).

While the underlying mechanism of exosome secretion remains poorly understood, an increase in exosome secretion has been associated with neoplastic transformation^{55, 56}. In order to demonstrate the capability to relate the cell secretion with genes expression, MYC⁵⁷ and OLIG2⁵⁸ were analyzed as they are key genes implicated in glioblastoma pathogenesis were tested. The exosome secretion rate of single cells was determined and categorized as low (<60) and high (>80) secretors. Random clones of low and high secreting single cells were then subjected to qPCR for MYC and OLIG2 expression. The results are show in **Figure 3.9**. A positive correlation was demonstrated between exosome secretion rate with MYC ($p = 0.0098$, **Figure 3.9 (a)**) and OLIG2 ($p = 0.0127$, **Figure 3.9 (b)**) expression. More importantly, the combination of MYC and OLIG2 gene expression established a higher correlation with the

exosome secretion rate than the expression of the individual genes alone (**Figure 3.9 (c)**). These results suggest that exosome secretion in glioblastoma is dictated by the physiologies mediated by MYC and OLIG2.

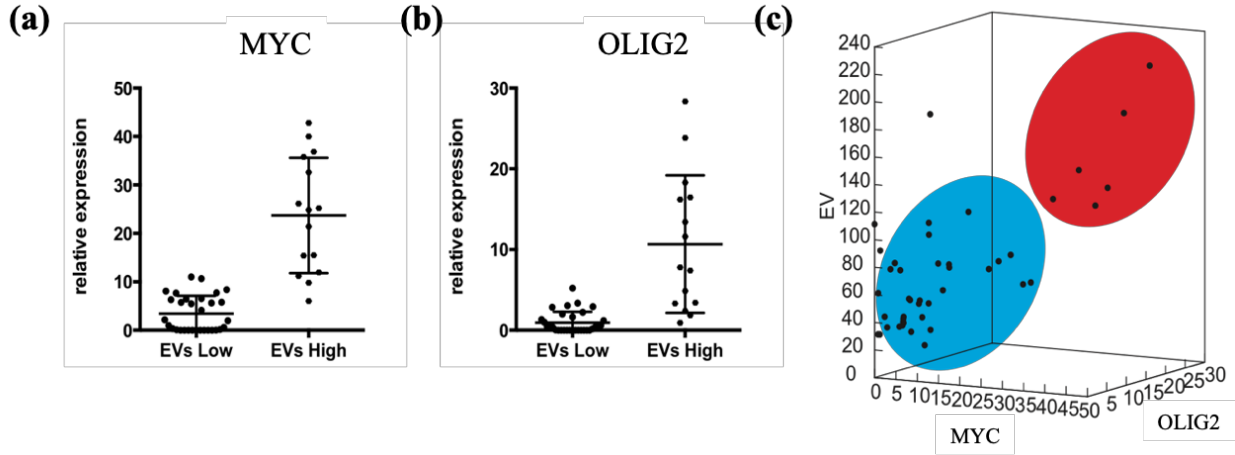


Figure 3.9 Relation between the exosome secretion rate and gene expression. (a) Correlation of MYC expression with exosome secretion ($p = 0.0098$). (b) Correlation of OLIG expression with exosome secretion ($p = 0.0127$). (c) Exosome secretion rate and expressions of MYC and OLIG show two distinct distributions of CMK3. The p-value was fitted by a linear line.

Not only can gene expression from single cells be studied, but exosome cargo analysis can be achieved by holistic secretion collection from the bottom. Exosomal miRNAs represent a particularly attractive bio-marker platform for ‘liquid biopsies’. miR-21 is a miRNA that is highly over-expressed in glioblastoma cells⁵⁹. The expression of miR-21 in glioblastoma mediates several essential oncogenic functions, including suppression of apoptosis, growth proliferation and tolerance of DNA damage. Droplet digital PCR (ddPCR) was used to investigate the copy number of miR-21 carried by exosomes secreted by each CMK3. Since the main purpose is to demonstrate the feasibility of investigating the exosome secretion rate and the miRNA carried by the exosomes as molecular cargos, we only choose 5 cells (two low-exosome secretion cells and 3 high exosome secretion cells) for analysis with the results shown in **Figure**

3.10. Interestingly, this preliminary result reveals positive correlations between the exosome secretion rate and the miR-21 copy numbers carried by the exosomes.

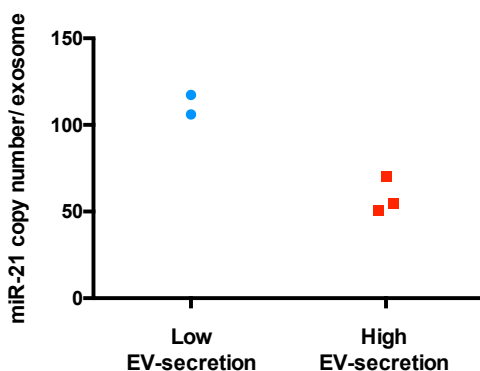


Figure 3.10 Exosomal miR-21 copy number per exosome in culture media measured by ddPCR and the exosome secretion rate from five single cells, demonstrating the versatility of the single cell assay

All the exosomes from single cells were lysed in 9 μ L lysis buffer (50 mM Tris HCl pH 8, 140 mM NaCl, 1.5 mM MgCl₂, and 250 μ L IGEPAL) containing 1 mg/ml BSA⁶⁰. Immediately before exosome lysing, 5 μ L/mL RNasin ribonuclease inhibitor (Promega, Madison, WI) was added to protect miRNA from RNases in the following cDNA synthesis reactions. Cells were lysed by gentle rocking at room temperature for 10 minutes. The lysed cells were maintained on ice before cDNA synthesis. cDNA synthesis was performed using the TaqManTM microRNA reverse transcription kit (ThermoFisher Scientific, Santa Clara, CA) per the manufacturer's instructions. cDNA was pre-amplified using the Taqman preamp master mix (ThermoFisher Scientific, Santa Clara, CA) per the manufacturer's instructions. Droplet digital PCR was performed to assess the expression of miR-21 using the QX200TM droplet digitalTM PCR system (Bio-Rad).

Each single cell was lysed in 11 μ L lysis buffer (50 mM Tris HCl pH 8, 140 mM NaCl, 1.5 mM MgCl₂, and 250 μ L IGEPAL) containing 1 mg/mL BSA⁶⁰. Immediately before cell

lysing, the RNAsin ribonuclease inhibitor (Promega, Madison, WI) was added to protect mRNA from RNases in the following cDNA synthesis reactions. Cells were lysed by gentle rocking at room temperature for 10 minutes. The lysed cells were maintained on ice before cDNA synthesis. cDNA synthesis was performed using the iScript advanced cDNA synthesis kit (Bio-Rad, Hercules, CA) per the manufacturer's instructions. cDNA was pre-amplified using the Taqman preamp master mix (ThermoFisher Scientific, Santa Clara, CA) per the manufacturer's instructions. Quantitative PCR was performed to assess the expression of OLIG2, MYC, 18S rRNA and GAPDH SsoAdvanced universal SYBR green supermix (Bio-Rad).

3.10 Summary

To summarize, a single-cell assay that offers time-lapsed studies, is non-invasive, and capable of performing quantitative analysis of molecular markers and vesicles secreted by single cells was presented. The device is simple, low cost, versatile, and has moderately high throughput to support different type single-cell studies.

First, this platform has been demonstrated to support excellent single cell viability with continuous data up to 96 hours. Second, this platform is also capable in quantifying the various exosome secretion rates of single cells under different in-vitro conditions. Third, exosome secretion and cargo can be related to corresponding single cell gene analysis by this platform.

This chapter is based on the following papers: Yu-Jui Chiu, Wei Cai, Yu-Ru V. Shih, Ian Lian, and Yu-Hwa Lo. A single-cell assay for time lapse studies of exosome secretion and cell behaviors, *Small*. 12 (2016) 3658-3666 and Wei Cai, Yu-Jui Chiu, Valya Ramakrishnan, Yi-Huan Tsai, Clark Chen, Yu-Hwa Lo, A single-cell translocation and secretion assay (TransSeA),

Lab on a Chip, 18 (20): 3154-3162, 2018. The dissertation author was the primary investigator and author of this paper.

Chapter 4 Bioparticle enrichment using mass transport via IR-induced differential evaporation rate

4.1 Overview

Sample enrichment is an important step in the work flow of biosensing for disease detection and numerous biological or clinical processes. Most current techniques require specially designed devices tailored to specific chemical or physical characteristics of the target objects to enrich or capture them from the sample, which may increase cost and limit the enrichment factor. In this chapter I will demonstrate a technique of using a CO₂ laser to induce mass transport to drive the targets towards the laser spot without requiring any device fabrication process or special reagents. To prove the concept, 1) fluorescein amidite (FAM) labeled single-strand DNA were determined to be enriched by more than 100,000-fold in less than 4 minutes; 2) the preliminary data of forming the aggregation by concentration was also presented; 3) the temperature profile, evaporation rate profile at the enriched area were measured, along with theoretical analyses and modeling, to monitor and understand the physical process. The method is not only simple and highly efficient, but also applicable to all types of biomolecules and bioparticles, promising a simple, cost effective and efficient solution for biological sample preparation for sensing, analytics, and diagnostics

4.2 CO₂ laser setup and enrichment effect

The CO₂ laser system is designed to generate the mass transport which enriches the solute in the liquid. **Figure 4.1** is a schematic illustrating the experimental setup. The heat source is a 10 W CO₂ laser (Universal Laser Systems ULR-10) with emission wavelength of 10.6 μm , having a nearly collimated output (4 mm beam width and 5 mrad divergence angle). The laser power is modulated at 20 kHz and the output power is controlled by the duty cycle ranging from 0 - 100%. To achieve the mass transport effect, we expand the laser beam by a 10 $\times$ beam expander composed of a pair of plano-concave (focal length -50 mm, diameter 1") and plano-convex (focal length 500 mm, diameter 1") zinc selenide (ZnSe) lenses with broadband (7 - 12 μm) antireflective coating. The expanded laser beam with 40 mm diameter is reflected by a 45° angle gold-coated mirror to direct the beam towards the bottom of the sample. The laser beam is then focused by a plano-convex (focal length 50 mm, diameter 1") ZnSe lens into a 30 μm spot on the bottom of the sample solution through a thin cover glass slide. The excitation source is the 488nm or 406nm laser fiber depending on the fluorescence properties of the solute or particles. The excitation laser is collimated and first reflected by the 505nm long pass dichroic mirror, then through the 20X objective lens. The light emission is collected back through the same objective lens, transmitted through the 505nm long pass dichroic mirror, and then detected by a CCD camera.

When the laser is turned on, the solute in the solution moves towards the laser spot by mass transport as illustrated in **Figure 4.2**.

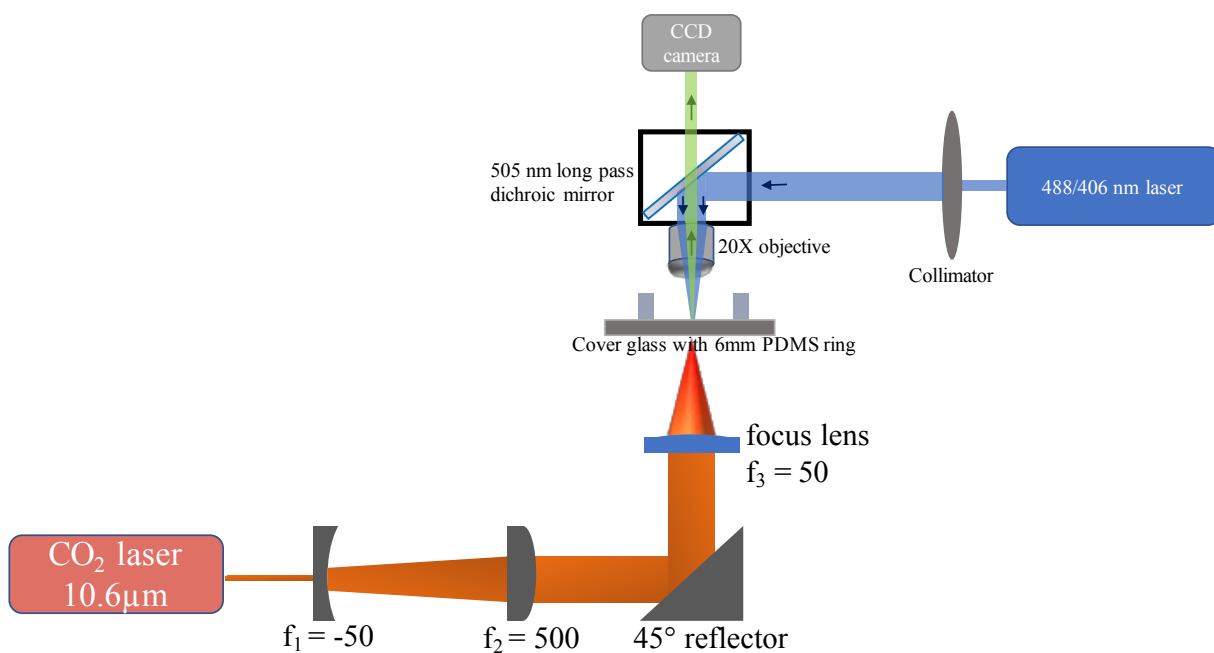


Figure 4.1 Schematic of the experimental setup

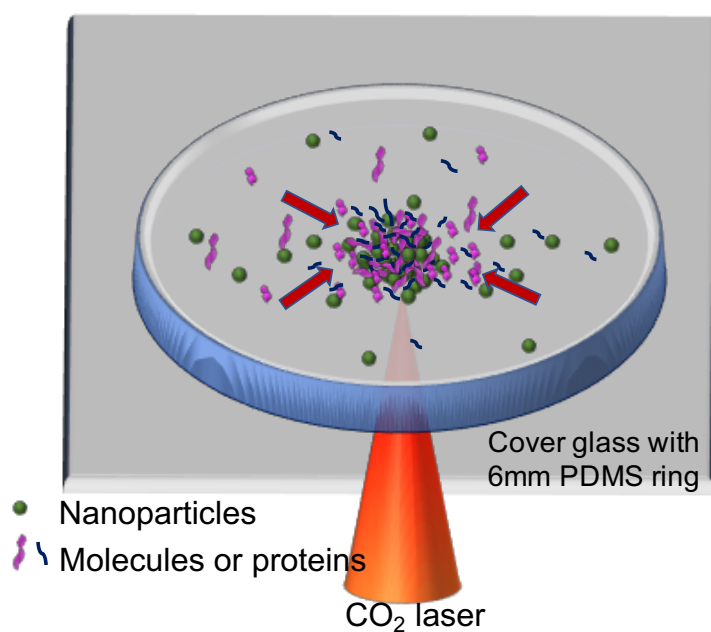


Figure 4.2 The operation principle of CO₂ laser induced enrichment

To demonstrate the mass transport effect induced by laser, an 8 μL sample containing 1 μM fluorescent ssDNA (single-stranded FAM labeled DNA, 90 nts in length,) was dispensed into the chamber with PDMS wall and a bottom surface composed of a cover glass. **Figure 4.3** shows the fluorescent intensity profile from the FAM labeled ssDNA over time. The enrichment begins at around 60 s after the laser irradiation, and the concentration increases exponentially because the mass transport effect becomes dominant to govern the travel of DNAs in the sample solution. The fluorescent intensity profile in **Figure 4.3 (a)** is plateaued due to the saturation of the CCD camera while the enrichment process continues. The estimated laser power absorbed by the sample is around 40 mW, giving rise to an evaporation rate of 1 $\mu\text{L}/\text{min}$. **Figure 4.3 (b)** shows representative images of the fluorescent intensity distribution after laser irradiation for 90 s, 120 s and 180 s, respectively.

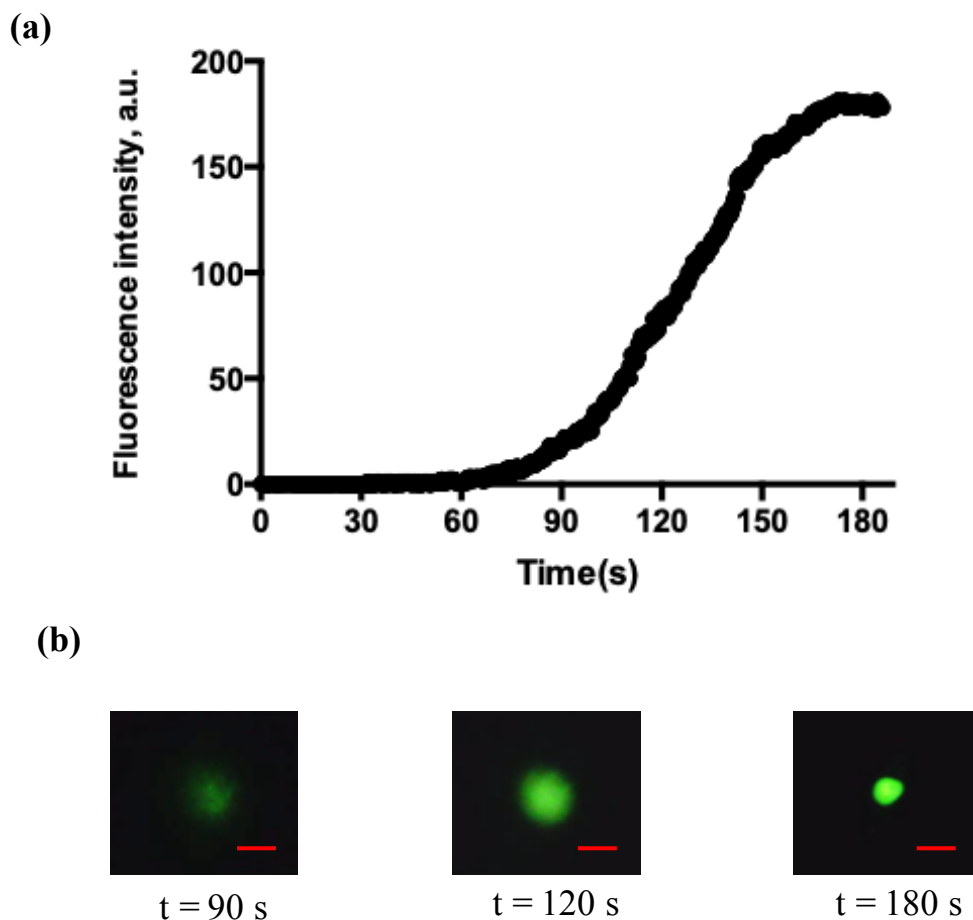


Figure 4.3 Fluorescent ssDNA enrichment profile. (a) Fluorescence intensity change over time. The intensity saturation after 150 seconds is due to the dynamic range limit of the CCD camera. (b) Representative images showing the spatial distribution of the fluorescent ssDNA at $t = 90$ s; $t = 120$ s and $t = 180$ s, respectively. The scale bar is $200\ \mu\text{m}$.

To quantitatively study the enrichment power of the method, a series of experiments with different initial DNA concentrations ranging from $100\ \text{nM}$ to $10\ \text{pM}$ in $8\ \mu\text{L}$ solution were performed. **Figure 4.4** shows the enrichment profile of each initial DNA concentration after 4 minutes of CO_2 laser irradiation. The camera exposure time was controlled and any nonlinear response of the CCD camera was removed to relate the camera output to the DNA concentration. The enrichment factor is obtained from the comparison between the peak intensity of the enriched sample and the background intensity of a pre-enriched sample of higher DNA

concentration. For instance, as the maximum fluorescent intensity for a 10 pM sample after enrichment matches that of a 1 μ M DNA sample without enrichment, we determine that the enrichment factor is 100,000. The intensity profile also produces the DNA concentration profile in the solution.

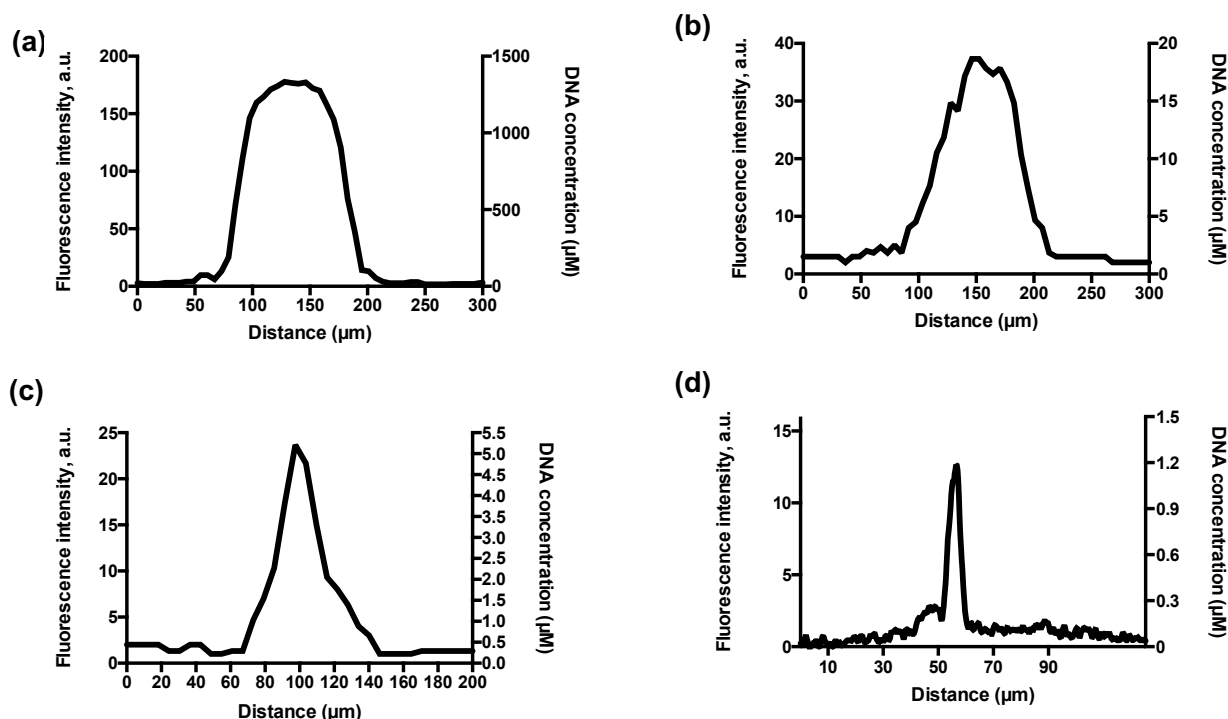


Figure 4.4 Fluorescent DNA profile with different initial concentrations (a) 100 nM, (b) 10 nM, (c) 100 pM, (d) 10 pM. All data are taken after 4 minutes of CO₂ laser irradiation. The y-axes show both the fluorescent intensity (left) from calibrated CCD output signal and the actual DNA concentration profile (right).

CO₂ laser induced enrichment has the following salient features suitable for sample enrichment for biosensing and biological sample processes: (1) the method works for particles of any size, shape, and physical and chemical properties, (2) the method can handle a wide range of initial particle concentration, (3) the method does not require any device processing such as formation of electrodes, microfluidic devices, or surface functionalization (e.g. control of hydrophobicity, surface charge control, specific capturing probes, etc.), and (4) having the CO₂

laser illuminate the sample solution from the bottom, the method offers a completely open platform, as opposed to a closed system found in most sample enrichment devices, to allow convenient monitoring and accessibility for changing, adding, or replenishing the sample solution any time during the action. The formation of aggregates is one of the examples to use this powerful enrichment method with **Figure 4.5 (a)** showing the concept of the aggregates. MCF7/GFP was cultured in exosome free medium in a 10 cm culture dish. The condition media was collected after 48 hours and cells number is around 4 million, based on our previous results, the exosome secretion rate of MCF7/GFP is ~ 60 exosome / (Cell*hour), so the estimated exosome number is around 10^9 exosome/mL. Then this condition media was incubated with 1.2 nM biotinylated anti-CD63 at 4°C for overnight to form biotinylated exosomes. 4 μ L biotinylated exosomes solution and 4 μ L 40 pM 25 nm streptavidin Q-dots are mixed and dispense on the device. The control experiment is 8 μ L Q-dots only. After 4 mins of CO₂ laser irradiation, as shown in **Figure 4.5 (b) - (c)**, Q-dots and exosomes in both solutions were enriched by the laser center. After turning off CO₂ laser, the Q-dots rapidly diffuse out in the control experiment (**Figure 4.5 (b)**). For a sample with biotinylated exosomes, the formation of aggregates causes only the unbound Q-dots to diffuse out. Therefore, fluorescence can still be observed 3 mins after the laser is turned off. (**Figure 4.5 (c)**).

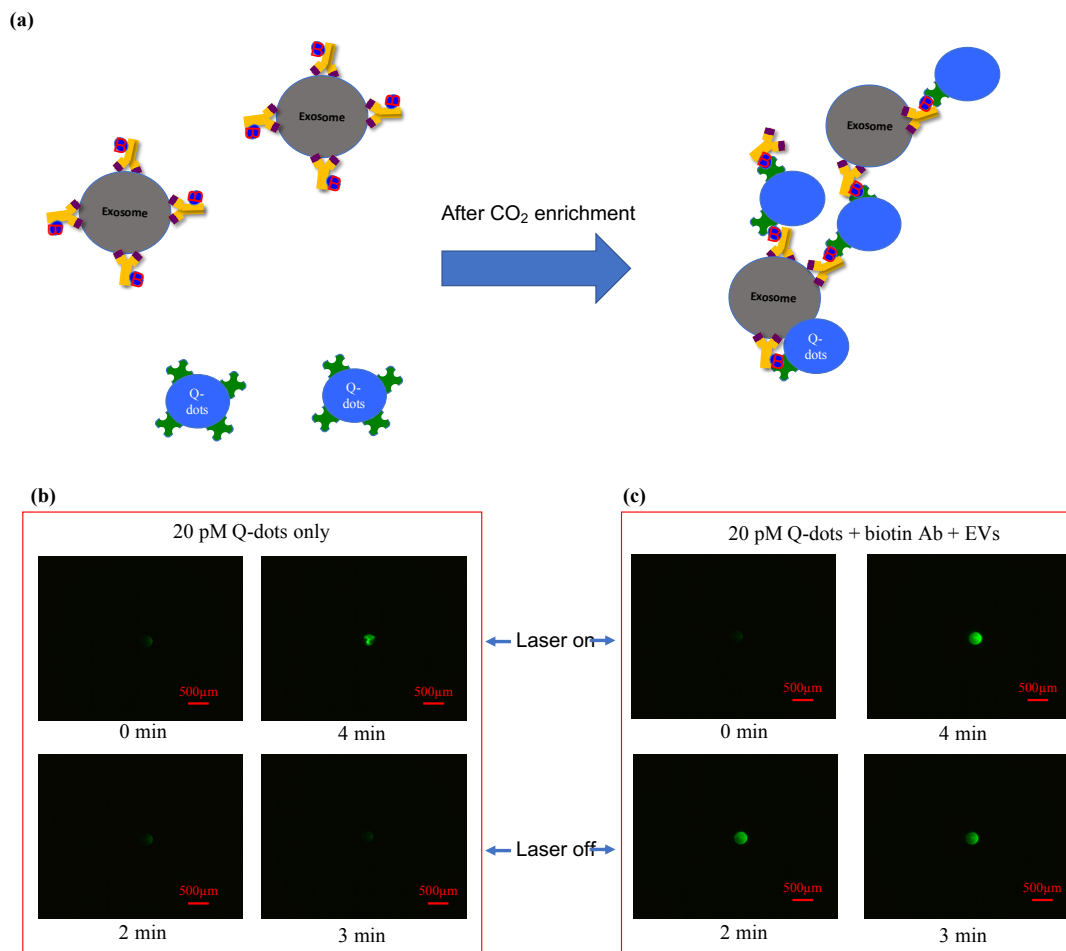


Figure 4.5 (a) The schematic of the formation of aggregates. (b) 20 pM streptavidin Q-dots enriched by CO₂ laser; (b) Aggregation formation with CO₂ laser

Although the laser-induced differential evaporation rates give rise to mass transport towards the laser spot, it is also accompanied with a temperature profile. While it is difficult to measure the evaporation rate profile, we can find such information from measurements of the temperature profile using a thermal imager (A325sc from FLIR). Furthermore, the temperature profile is of interest for biological samples such as DNAs and proteins. In our experiment, 40 mW CO₂ laser power that is focused to a 30 µm spot raises the spot temperature from 25 °C to 58.5 °C in 10 seconds. At the same time the effects of diffusion and convection increase the temperature of the surrounding area (spanning a diameter of 3 millimeters) to 42 °C, giving rise

to a net evaporation rate of 1 $\mu\text{l}/\text{min}$. **Figure 4.6 (b)** exhibits how the temperature profile changes during the first 60 seconds of CO_2 laser irradiation. After 60 seconds, the temperature profile reaches steady state.

Using the temperature profile (**Figure 4.6**) and the relation between temperature and evaporation rate, the evaporation rate profile can be produced (**Figure 4.7**).

Water evaporation rate per area is calculated as:

$$E = \theta * (X_s - X) \quad (1-1)$$

$$\theta = 25 + 19 * v \quad (1-2)$$

where θ is the evaporation coefficient, v is the velocity of air above the sample surface which we assumed to be 0 in our calculation. X is the humidity ratio in air⁶¹. Based on the Ideal Gas Law, the humidity ratio can be represented as

$$X = 0.62198 * P_w / (P_a - P_w) \quad (1-3)$$

P_w is the partial pressure of water vapor in moist air and P_a is atmospheric pressure of 101,325 Pa. X_s is the maximum humidity ratio of humidity saturated air of the same temperature as the sample surface.

$$X_s = 0.62198 * P_{ws} / (P_a - P_{ws}) \quad (1-4)$$

According to Antoine equation, saturated pressure of water vapor can be expressed as:

$$P_{ws} = e^{(77.345 + 0.0057 * T - 7235/T)} / T^{8.2} \quad (1-5)$$

The humidity is

$$\phi = \frac{P_w}{P_{ws}} * 100\% \quad (1-6)$$

The evaporation rate reaches the maximum value at the center area, due to its highest surface temperature. The following Computational Fluid Dynamics (CFD) computations will

demonstrate that the mass transport via the differential evaporation rate is key to sample enrichment. It should also be noted that the evaporation rate is highly correlated with the humidity in the air phase. As humidity decreases, the evaporation rate increases and thus subsequently affects the enrichment process.

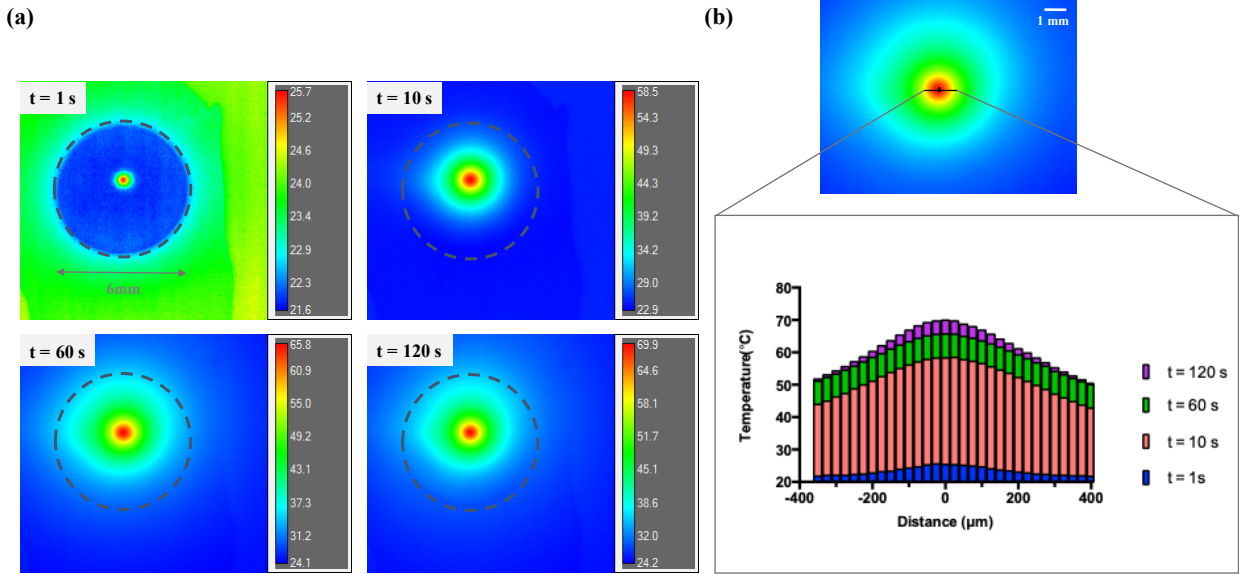


Figure 4.6 (a) Temperature heat map for different CO₂ laser irradiation time periods: 1 s, 10 s, 60 s and 120 s. (b) Temperature profiles under different durations of CO₂ laser irradiation.

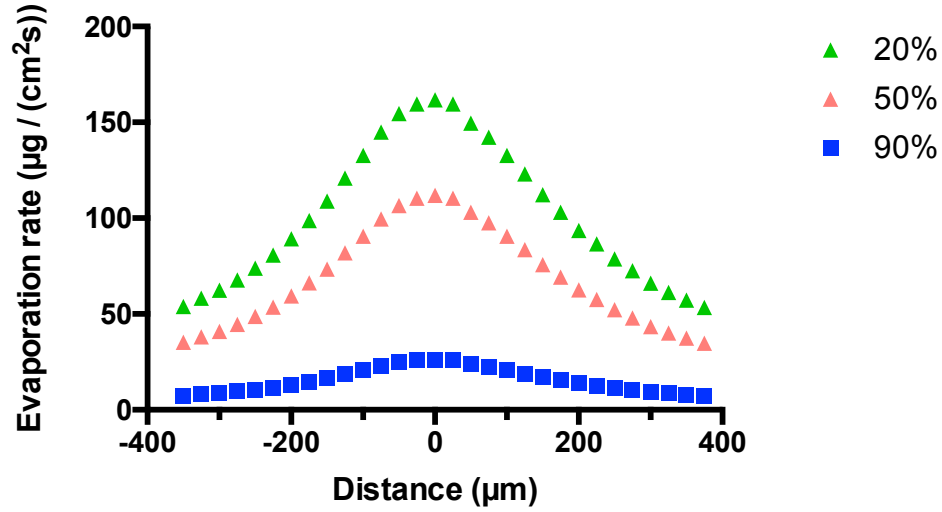


Figure 4.7 Calculated sample evaporation rate profile at 20%, 50%, 90% relative humidity using the temperature profile at 120 s in Figure 4.5

4.3 CFD Simulations

To elucidate the experimental results of laser-induced enrichment, CFD simulations were performed to provide insights on the fluid flow, which is critical to the differential mass transport by CO₂ laser induced evaporation.

4.3.1 Governing Equations

The liquid evaporation with laser heating can be modeled with two-phase laminar flow using Arbitrary Lagrangian Eulerian moving mesh method in the axisymmetric three dimensional cylinder coordinate.

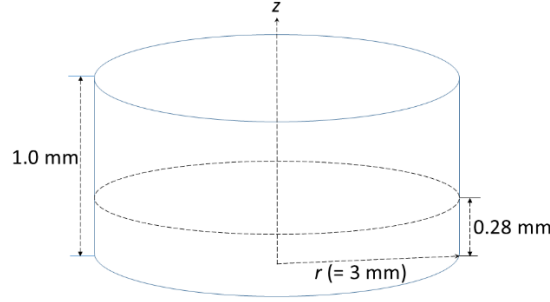


Figure 4.8 Schematics of the water-vapor system. The laser is applied at the central of the bottom surface.

The governing equations incorporate mass, momentum and heat transfer coupled with interfacial evaporation with the aid of local laser-heating. The Navier-Stoke equation is solved along with continuity equation of compressibility to compute velocities of laminar flows for water liquid phase and vapor phase,

$$\rho \left[\frac{\partial \mathbf{u}}{\partial t} + (\mathbf{u} \cdot \nabla) \mathbf{u} \right] = \nabla \cdot \left[-p \cdot \mathbf{I} + \mu (\nabla \mathbf{u} + \nabla \mathbf{u}^T) - \frac{2}{3} \mu (\nabla \cdot \mathbf{u}) \mathbf{I} \right] + \mathbf{F} \quad (3-1)$$

$$\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho \mathbf{u}) = 0 \quad (3-2)$$

where ρ is the density; μ represents the viscosity; p is the pressure; and $\mathbf{F}$ is the volume force arisen from the gravity. The temperature profile inside the water phase and the vapor phase are simulated with the heat transfer equation, which consists of heat convection and conduction,

$$\rho C_p \frac{\partial T}{\partial t} + \rho C_p \mathbf{u} \cdot \nabla T + \nabla \cdot (-k \nabla T) = 0 \quad (3-3)$$

where T is temperature, C_p represents heat capacity; and k stands for water heat conductivity. The momentum and heat transfer equations are solved for coupled gas and liquid phases. The mass transfer inside the vapor phases is expressed in terms of convection and diffusion,

$$\frac{\partial c}{\partial t} + (\mathbf{u} \cdot \nabla) c - \nabla \cdot (D \nabla c) = 0 \quad (3-4)$$

where c and t stand water molar concentration and time respectively; $\mathbf{u}$ is the velocity, obtained from momentum equation; and D is the diffusion coefficient.

4.3.2 Initial Conditions

The initial condition for the vapor domain is the ambient temperature, $T_0 (= 293.15 \text{ K})$, and the relative humidity X_h is 30%, then initial water concentration, $c_{w0}^v = c_{w0}^{sat} * X_h$ is calculated according to ideal gas equation of state. The initial saturated concentration c_{w0}^{sat} at the ambient temperature is also calculated as, P_{sat}/RT , where the saturated steam pressure is estimated with the Antoine equation. All other physical properties such as density, viscosity and diffusivities are evaluated at ambient temperature and pressure ($p_0 = 1 \text{ atm}$). The initial velocities in the vapor and the liquid phase are assigned as zero.

4.3.3 Boundary Conditions

In the boundary conditions, for the momentum transfer in the vapor phase, the open boundary with constant pressure is given at the top part of vapor phase,

$$p = 0 \text{ atm, at } z = 0.6 \text{ mm} \quad (3-5)$$

where p is the dynamic pressure, total pressure $p_{total} = p_0 + p$. At the PDMS-water interface, a slip wall is adopted and a constant PDMS-air-water contact angle, $\theta_w = 110^\circ$, is assigned to compute surface effect,

$$\gamma_{sl} - \gamma_{sv} = \sigma_{lv} \cos \theta_w \quad (3-6)$$

where γ_{sl} , γ_{sv} and σ are surface energy density for surface-lipid, surface-vapor, and surface tension for liquid-vapor interfaces respectively. In the simulation, the temperature-

dependent σ_{lv} was adopted from literatures. The whole PDMS side wall is slippery with zero normal velocity; and the bottom of container is simply no flow conditions.

$$\mathbf{n} \cdot \mathbf{u} = 0, \text{ at } r = 3.0 \text{ mm} \quad (3-7)$$

$$\mathbf{u} = \mathbf{0}, \text{ at } z = 0 \text{ mm} \quad (3-8)$$

where r is the cylindrical r -coordinate.

For the mass transfer in the vapor phase, the outer condition is given at the top part of the vapor phase ($z = 0.6 \text{ mm}$).

$$c = c_{w0}^v, \text{ if } \mathbf{n} \cdot \mathbf{u} < 0 \quad (3-9)$$

$$-\mathbf{n} \cdot \mathbf{q} = 0, \text{ if } \mathbf{n} \cdot \mathbf{u} \geq 0 \quad (3-10)$$

At the lipid-vapor interface, the mass flux condition $\dot{m}_e$ is applied,

$$-\mathbf{n} \cdot \mathbf{q} = \dot{m}_e \quad (3-11)$$

$$\dot{m}_e = k_e(c_w^{sat} - c_w^v) \quad (3-12)$$

where c_w^{sat} and c_w^v represent instantaneous saturation water concentration and water vapor concentration respectively; and $k_e (=0.02 \text{ (l/s)})$ stands for the mass transfer rate between liquid and vapor phases. At the PDMS side wall, there is no mass flux,

$$\frac{\partial c}{\partial z} = 0, \quad \text{at } r = 3 \text{ mm and } z = 0 \text{ mm (if interface touches bottom)} \quad (3-13)$$

For the heat transfer, the outer boundary is adopted for the upper part of the vapor phase ($z = 0.5 \text{ mm}$),

$$T = T_0, \text{ if } \mathbf{n} \cdot \mathbf{u} < 0 \quad (3-14)$$

$$-\mathbf{n} \cdot \mathbf{q} = 0, \text{ if } \mathbf{n} \cdot \mathbf{u} \geq 0 \quad (3-15)$$

At the lipid-vapor interface, the heat conservation is reserved,

$$\rho C_p \frac{\partial T}{\partial t} + \rho C_p \mathbf{u} \cdot \nabla T + \nabla \cdot (-k \nabla T) = Q_e \quad (3-16)$$

$$Q_e = -h_e m_e w_e \quad (3-17)$$

where h_e is water latent heat (J/kg), which is determined by the instantaneous temperature. At the bottom part of the water phase, there is heat supply ($Q_p = 0.04 \text{ W}$) at the central part of the bottom surface,

$$-\mathbf{n} \cdot \mathbf{q} = Q_p, \text{ at } r \leq 100 \text{ } \mu\text{m}, z=0 \quad (3-18)$$

It should be noted although Q_p is assigned at the bottom of the surface, if the grid mesh is considered, Q_p is applied on a grid with the length of $15 \text{ } \mu\text{m}$.

For the other area of the bottom surface (at $z = 0.0 \text{ mm}$ and $r > 150 \text{ } \mu\text{m}$) or the PDMS side wall ($r = 3.0 \text{ mm}$), heat insulation condition is applied.

$$-\mathbf{n} \cdot \mathbf{q} = 0, \quad (3-19)$$

The partial differential equations were solved with COMSOL multiphysics software (Version 5.2, COMSOL Inc. USA).

The simulation results show two main effects that cause water to flow: thermocapillary convection and differential evaporation. Previous studies showed that natural convection is strongly suppressed in comparison with thermocapillary convection for a small system with length less than a few millimeters⁴⁶. Thermocapillary convection is resulted from a surface tension gradient due to the temperature profile in the fluid since surface tension is reduced with increasing temperature.

Figure 4.9 shows the simulated water velocity profile at different times (12 s and 322 s) after the CO₂ laser is turned on. At positions far from the center, water flows from the PDMS boundary of the reservoir towards the center by taking a path near the bottom surface. When the flow reaches some distance from the center, the flow direction turns upward, driven by two mechanisms: thermocapillary effect and differential evaporation. The thermocapillary effect circulates a part of the flow back by taking a path near the liquid surface, forming a convection

flow. On the other hand, another part of the flow that is very close to the center (e.g. within 100 μm from the center) takes a different path than the thermocapillary convection flow. Instead of circulating back, it reaches the liquid surface and evaporates.

The flow velocity profile helps envision how particles behave in the liquid. The particles will follow the flow path and, at the same time, experience random (Brownian) motions. They also experience interparticle interactions under certain concentration and local environment. Ignoring the Brownian motions and interparticle interactions, particles will be either accumulated at the surface where water evaporates or circle around following the path of thermocapillary convection. For those particles circulating around, over time their Brownian motions can bring them to the flow path that ends at the center area dominated by evaporation. Once the particles are accumulated at the surface, they are trapped by the differential evaporation effect, as calculated in **Figure 4.10**. Therefore, the interplays among differential evaporation, thermocapillary convection, and Brownian motions can yield a very high enrichment factor.

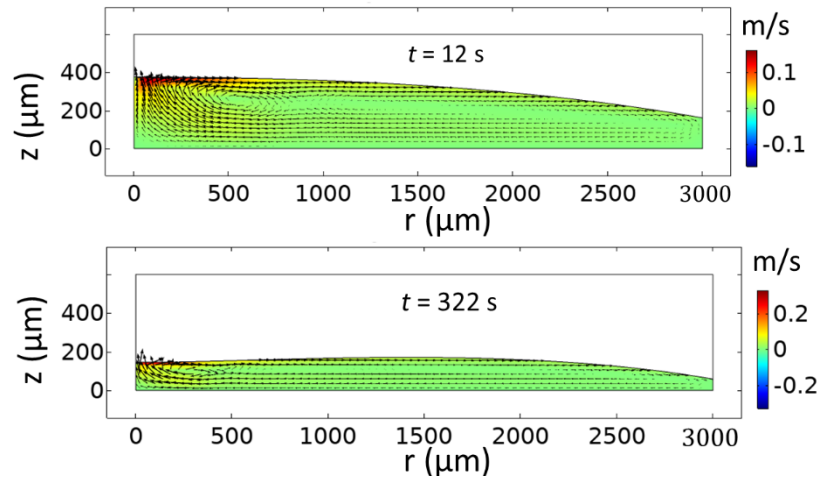


Figure 4.9 Velocity profile at $t = 12$ s and 322 s after the CO_2 laser focused to the center ($r = 0$ μm) position is turned on. The velocity profile shows the differential evaporation effect that terminates the liquid flow at the surface near the center position and the thermocapillary effect that creates a convection flow.

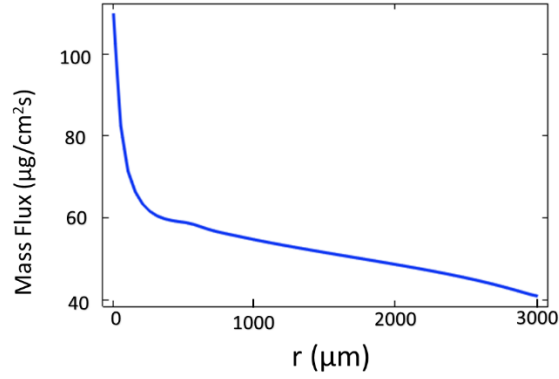


Figure 4.10 Mass Flux at water-air interface as a function of r at $t = 322$ s.

For a detailed model for enrichment of a particular type of particles such as DNA molecule, we need to take into account the specific properties of the particles⁶² such as the Brownian (diffusion) force, drag force, gravity, and molecular interactions including Lennard-Jones, Coulombic and thermophoresis forces. These effects, together with the aforementioned differential evaporation and thermocapillary effect that are universal to all particles in our experimental setup, will determine the details of the enrichment factor and particle behaviors.

4.4 Desalination

Since the enrichment would apply to all the solute in the liquid, the ionic strength may change during the process. However, most molecular tests require specific buffer and salt concentration. Therefore, conditioning the sample before the bioprocess is very important.

4.4.1 Device design and characterization

The purpose of this device is for selective removal or addition of components while minimizing dilution of sample and loss of target molecules through the exploitation of the

considerable difference between the diffusivities of salt and macromolecules. A schematic of the device design is shown in **Figure 4.11**. The chamber is prefilled with the sample and DI water is subsequently flowed through the channel at a speed of 1 - 10 cm/s. By controlling the duration of water flow, we can regulate the degree of desalting and removal rate of small molecules by diffusion.

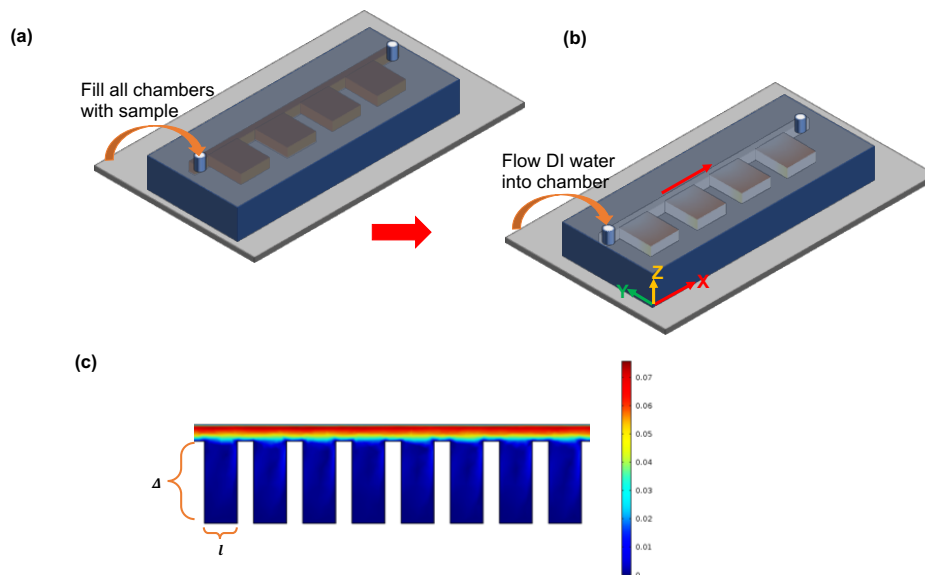


Figure 4.11 Schematic illustration of device design and operation. (a) All chambers are first filled with the sample. (b) Flow DI water through the channel to establish a concentration gradient between the chamber and the channel for each component in the sample, which drives diffusion. If desired, components lacking in the original sample can be introduced to the chamber by establishing a reverse concentration gradient between the channel and the chamber. (c) Simulated fluid velocity distribution, indicating the samples in each chamber are in the dead volume (zero velocity). Color scale bar: flow speed in m/s.

All chambers are 200 μm wide and the flow channel is 100 μm wide; and both structures are 200 μm high. We fabricated three different chamber depths: $\Delta = 250 \mu\text{m}$, $\Delta = 350 \mu\text{m}$, $\Delta = 500 \mu\text{m}$. For sample desalting experiment, the chambers were first filled with 1.5 nM fluorescein sodium salt (Sigma - Aldrich, F6377) and then the channel was flowed with DI water at 10 $\mu\text{L}/\text{min}$. The simulated flow velocity profile by COMSOL is shown in **Figure 4.11 (c)**. As the

fluorescein began to diffuse out of the chamber, the integrated fluorescent intensity of fluorescein sodium salt in each chamber decays exponentially over time. The curve can be fitted by a characteristic time constant till the intensity reaches the camera noise floor, as shown in **Figure 4.12 (a)**.

It was noted that experimentally we observed that the flow velocity profile was extended into each chamber by as deep as 100 μm , which somewhat deviated from the simulation results for an ideal laminar flow. However, below this flow penetration depth, both experiment and simulation showed the true “dead volume” where the flow velocity was zero. Therefore, we confirmed that in positions deeper than 100 μm in the chamber, the only mechanism for particle movement was via diffusion.

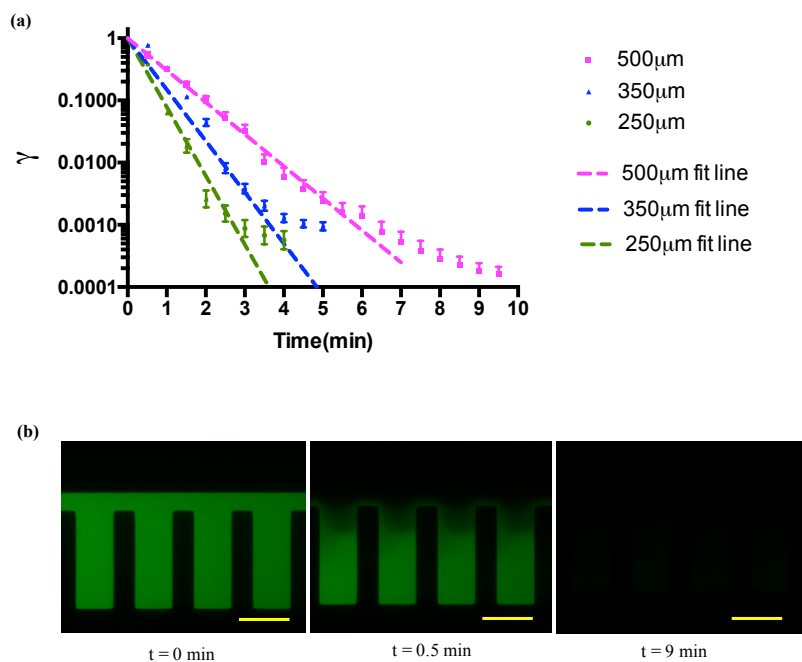


Figure 4.12 (a) Time dependent fluorescein sodium salt concentration profile for different chamber depths: $\Delta = 250 \mu\text{m}$, $\Delta = 350 \mu\text{m}$, $\Delta = 500 \mu\text{m}$. The dashed line represents the fit line by an exponential function with a characteristic time constant. (b) Time lapse fluorescence image for 500 μm deep chambers. The scale bar is 250 μm .

To demonstrate the capability for selective removal of fast diffusive objects while keeping the slowly diffusive objects in the chamber, we measured the time dependent fluorescent intensity of different particles (fluorescein sodium salt, 40 nm beads, 25 nm quantum dots, and fluorescent single-stranded FAM labeled DNA, 90nt s in length) in a 500 μm deep chamber. The time-dependent fluorescent intensities for the respective particles remaining in the chamber are shown in **Figure 4.13 (a)**. Figure **4.13 (b)** shows the superimposed images for 40 nm beads (red) and fluorescein sodium salt (green). In 3 minutes, nearly all salt has diffused out of the chamber while greater than 80% nanobeads remain in the chamber. The process has produced a reliable and predictable concentration for specific objects in the sample, as predicted by the closed form solution of the diffusion equation described in the next section.

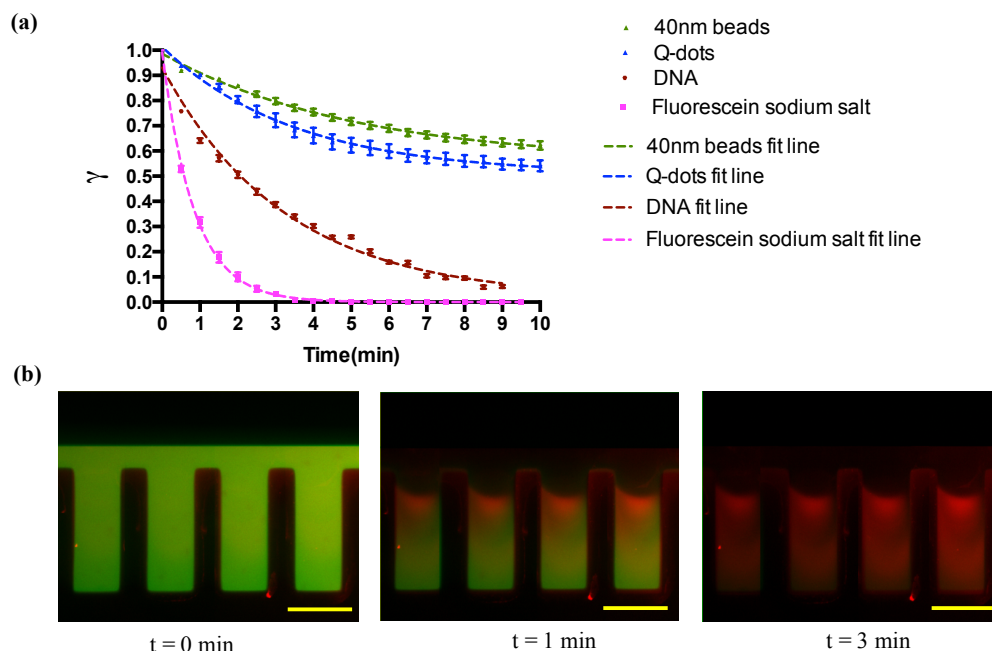


Figure 4.13 (a) Time dependent concentration for 40 nm fluorescent bead, 25 nm Q-dots, fluorescent ssDNA (single-stranded FAM labeled DNA, 90nt s in length,) and fluorescein sodium salt in 500 μm chamber design. γ is the fraction of particles in the chamber. (b) Time lapse fluorescent image for salt (green) and 40 nm beads (red). The scale bar is 250 μm . At 0 min, the fluorescence was dominated by that of fluorescent salt because of its much higher concentration. Over time, nearly all salt diffused out and only 40 nm beads remained in the chamber.

This device can also be used to add components to the sample without increasing the overall sample volume. To demonstrate this function, we reversed the process by flowing 1.5 nM fluorescein sodium salt at 1 $\mu\text{L}/\text{min}$ into the chamber. The chamber was prefilled with fluorescent ssDNA. As the fluorescein began to diffuse into the chamber, the integrated fluorescence intensity in the chamber increased. The results are shown in **Figure 4.14**.

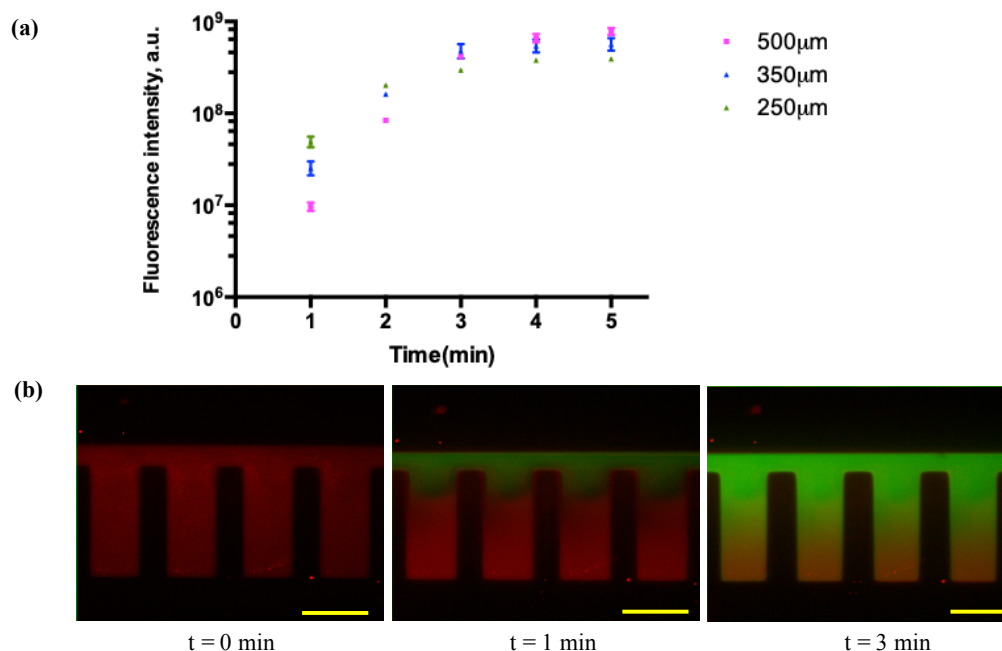


Figure 4.14 Demonstration of introducing specific components into the sample in a controllable manner without increasing the sample volume. (a) Time dependent concentration of fluorescein sodium salt added to the chamber of different depths: $\Delta = 250 \mu\text{m}$, $350 \mu\text{m}$, $500 \mu\text{m}$. (b) Time lapse fluorescent images by adding salt into fluorescent ssDNA solution. Fluorescein sodium salt is in green color and fluorescent ssDNA is in red color. The scale bar is $250 \mu\text{m}$.

4.4.2 Physical Model

In this section we mathematically formulate the problem and obtain analytical solutions for the concentration profiles of particles with different diffusivity. The model provides a simple and accurate solution showing dependence of the solutions on all physical parameters explicitly.

The continuity equation for particles within a chamber in figure 1 can be written as,

$$\frac{\partial n(x,y)}{\partial t} = -\frac{\partial}{\partial x}F_x - \frac{\partial}{\partial y}F_y \quad (4-1)$$

where $n(x, y)$ is the particle concentration in each chamber ($0 \leq x \leq l$, $0 \leq y \leq \Delta$)

Assuming the sample in the chamber is in the dead volume of a laminar flow system, we have $v_x = 0, v_y = 0$ within the chamber. Therefore, only the diffusion terms contribute to the particle flux, and (4-1) can be simplified into (4-2) since the chamber geometry also produces the condition, $\frac{\partial}{\partial x} = 0$.

$$\frac{\partial n(t,y)}{\partial t} = D \frac{\partial^2}{\partial y^2} n(t, y) \quad (4-2)$$

The following boundary conditions apply to the problem:

$$\frac{\partial n(t,y=0)}{\partial y} = 0 \quad (4-3-A)$$

$$n(t, y = \Delta) = 0 \quad (4-3-B)$$

Here $y = 0$ is at the end of the chamber and $y = \Delta$ is at the exit of the chamber, with Δ being the chamber depth. (4-3-B) is true when the DI water flow speed in the channel is much greater than the diffusion velocity such that particles exiting the chamber are immediately removed by the flow, leaving the particle concentration at the chamber exit to be nearly zero.

For the initial condition, we assume a uniform concentration profile within the chamber before the experiment starts,

$$n(t = 0, y) = n_o[u(y) - u(y - \Delta)] \quad (4-3-C)$$

$u(y)$: unit step function

n_o : original particle concentration in the sample.

$t = 0$ (time when the water flow starts)

We assume a solution can be written in the form of $f(t)g(y)$. Substituting $f(t)g(y)$ into (4-2), we obtain

$$\frac{1}{f} \frac{df(t)}{dt} = \frac{\left[D \frac{d^2 g}{dy^2} \right]}{g(x,y)} = -K$$

The solutions of the above equation can be written as,

$$f(t) = f(0)e^{-Kt}$$

$$g(y) = \cos \left(\sqrt{\frac{K}{D}} y \right).$$

Here we drop the other possible solution $\sin \left(\sqrt{\frac{K}{D}} y \right)$ for $g(y)$ because it does not satisfy the boundary condition (4-3-A).

For $g(y) = \cos \left(\sqrt{\frac{K}{D}} y \right)$ to satisfy the boundary condition (3-B), we require

$$\sqrt{\frac{K}{D}} \Delta = \frac{\pi}{2} (2n + 1).$$

The allowed values for K are,

$$K_n = D \left[\frac{\pi}{2\Delta} (2n + 1) \right]^2 \quad n = 0, 1, 2, \dots \quad (4-4)$$

Then the solution $f(t)g(y)$ can be written as,

$$f(t)g(y) = f(0)e^{-K_n t} \cos \left(\frac{\pi}{2\Delta} (2n + 1)y \right) \quad (4-5)$$

The general solution for (2) can be represented as,

$$n(t, y) = \sum_0^\infty A_n e^{-K_n t} \cos \left[\frac{\pi}{2\Delta} (2n + 1)y \right] \quad (4-6)$$

Using the initial condition (4-3-C), we find

$$A_n = \frac{n_o \int_0^\Delta \cos \left[\frac{\pi}{2\Delta} (2n+1)y \right] dy}{\int_0^\Delta \cos^2 \left[\frac{\pi}{2\Delta} (2n+1)y \right] dy} = n_o \frac{4}{(2n+1)\pi} (-1)^n \quad (4-7)$$

Substitute (4-7) into (4-6), we have the solution for (4-2) that satisfies the boundary and initial conditions:

$$n(t, y) = n_o \sum_0^{\infty} \frac{4}{(2n+1)\pi} (-1)^n e^{-\left[\frac{\pi}{2\Delta}(2n+1)\right]^2 Dt} \cos \left[\frac{\pi}{2\Delta} (2n+1)y \right] \quad (4-8)$$

Integrating (4-8) over the depth (Δ) of the chamber,

$$N(t) = \int_0^{\Delta} n(t, y) dy = n_o \Delta \sum_0^{\infty} \frac{8}{(2n+1)^2 \pi^2} e^{-\left[\frac{\pi}{2\Delta}(2n+1)\right]^2 Dt} \quad (4-9)$$

The fraction of the component left in the sample after a time duration “t” becomes,

$$\gamma = \frac{N(t)}{n_o \Delta} = \sum_0^{\infty} \frac{8}{(2n+1)^2 \pi^2} e^{-\left[\frac{\pi}{2\Delta}(2n+1)\right]^2 Dt} \quad (4-10)$$

For an approximate solution, we keep only the $n = 0$ term in (4-10) because after a long enough time period that satisfies the condition $\left[\frac{\pi}{2\Delta}\right]^2 Dt > 1$, all higher order ($n > 0$) terms decay to a much lower value than the $n = 0$ term. Thus,

$$\gamma = \frac{N(t)}{n_o \Delta} \sim \frac{8}{\pi^2} e^{-\left[\frac{\pi}{2\Delta}\right]^2 Dt} \quad \text{when } \left[\frac{\pi}{2\Delta}\right]^2 Dt > 1 \quad (4-11)$$

Therefore, from the fraction of specific component left in the chamber after a certain time period, we can determine the diffusivity of the component. The diffusion coefficients, calculated from **Eq. (4-11)** and listed in **Table 4.1**, fit the experimental data in **Figure 4.12 (a)** and **Figure 4.13 (a)** very well.

Table 4.1 Diffusion coefficient of different solutes

Solute	D(cm ² /s)
Fluorescein sodium salt	1.99*10 ⁻⁵
ssDNA (90nt s)	5.19*10 ⁻⁶
Q-dots (25 nm)	4.83*10 ⁻⁶
40nm beads	3.32*10 ⁻⁶

4.5 Summary

In this chapter, a CO₂ laser-induced enrichment method was introduced that is applicable to numerous bioparticles regardless of their physical and chemical nature. Induced by evaporative mass-transport, the enrichment technique is capable of capturing particles over an extensive area and driving them to a single spot at the laser focus. It has been demonstrated that using roughly 40 mW from a CO₂ laser, an enrichment factor as high as 100,000 can be achieved in 3 - 4 minutes. The temperature at the laser spot could be monitored and adjusted while maintaining an enrichment profile, such that temperature sensitive protocols can still be achieved. The ease and flexibility of IR-induced enrichment permits integration into a multitude of detection systems to support multi-dimensional biosensing and orthogonal tests for in-vitro diagnosis. The aggregation formation was demonstrated to show the capability of sample purification and quantification. Also, a microfluidic device to condition liquid samples using the differential diffusivity was demonstrated. The technique helps us achieve desired environments for testing of target particles from biofluids. The method is particularly suitable for

removing/adding components to adjust the ionic strength and buffer condition for downstream analysis as ions usually have higher diffusivities than the bio-makers of interest such as exosomes, extracellular vesicles, proteins and DNAs. Excellent agreement was found between the physical model and the experiment, making the results highly reliable and predictable. Thus, the method can be an effective means to measure the diffusivity of objects in the sample, which offers valuable insight for the physical properties of macromolecules such as their radius of hydration and geometry in different microenvironments.

This chapter is based on the following in submitting papers: Wei Cai, Yi-Huan Tsai, Edward Wang, Ping-Wei Chen, Lennart Langouche, Chuan Lu, Tao Wei, Yu-Hwa Lo, “Bioparticle enrichment using mass transport via IR-induced differential evaporation rate” and Wei Cai, Edward Wang, Ping-Wei Chen, Yi-Huan Tsai, Lennart Langouche, Yu-Hwa Lo, “A microfluidic design for desalination and selective removal and addition of components in biosamples”. The dissertation author was the primary investigator and author of these papers.

Chapter 5 Conclusion

This thesis presented a single-cell assay and CO₂ induced enrichment for analysis of single cells secretion.

The single-cell assay consists of a polydimethylsiloxane (PDMS) array of microwells fabricated by direct lithographic lift-off process and a supporting layer at the bottom. Each well in the microarrays is 25 μm to 50 μm diameter for different size of cells, and the center-to-center distance between two wells is 400 μm . The supporting layer at the bottom can be a standard tissue culture plates or submicron pore sized filter.

To load single cells on the chip, cells suspended in the culture medium are dispensed on the chip by direct pipetting, followed by a low-speed centrifugation process that guides the cells into the wells. Any cells outside the wells are flushed away by gently pipetting the culture medium over the surface of the slightly tilted chip or by removing the PDMS mesh on the culture dish after the cells are attached to the bottom. With proper control of the cell concentration in the medium, over 70% of populated wells contain a single cell.

To collect secretion from single cells, a glass slide with immobilized antibodies (e.g. anti CD63 antibody) is placed on top of the cell culture chip or the holistic collection can be achieved by placing the chip with the filter on a 1536-well plate. Using this assay, exosome secretion from different cells were demonstrated. The results shown that MCF7 cells and MDA-MB-231 cells have a similar CD63 positive exosome secretion rate of 60 - 65 exosomes per hour, and MCF10A cells secrete 2.8X more CD63 positive exosomes than MCF7. A glioblastoma patient derived cell line, CMK3, shows a much broader distribution of exosome secretion rate than the aforementioned cell lines. Over a 3-hour period, the number of exosomes secreted by an

individual CMK3 cell varies from less than 10 exosomes to nearly 200 exosomes. By changing different secondary/biotinylated antibodies, the expression level of different surface proteins on those CD63 positive exosomes were investigated. Exosomes secreted by MDA-MB-231 tend to have higher expression level of CD9 and CD81 than MCF10A. While antibody-specific exosome secretion rate is characterized by the cover slides on the top, a long-term exosomes collection from single cells can also be achieved in the wells through the thin filter non-invasively. Using this technique, microRNAs carried by exosomes can be quantified using droplet digital PCR. The preliminary results shown positive correlations between miR-21 copy numbers carried by exosomes and the secretion rate of exosomes.

Another salient feature of this single cell assay is its open-well design, making it easy for cell pickup with standard tools such as pico-pipette for downstream analysis. By using the pico-pipette to selectively pick up single cells according to their exosome secretion profiles and conducting single cell qPCR analysis, the positive correlations between exosome secretion rate and expressions of MYC and OLIG2 genes for CMK3 cells were realized.

Next, a biosample enrichment was introduced. This method utilizes differential mass transport by a CO₂ laser induced evaporation. Utilizing the strong water absorption at the CO₂ wavelength (10.6 μ m), a differential evaporation rate over the sample formed with the highest evaporation rate at the laser focal spot. The differential evaporation rate generates a net flow of any suspended particles due to the effect of mass transport, which exceeds any diffusion effect due to thermal gradient. As a result, all suspended particles in the sample regardless of position or solubility are driven towards the area defined by the CO₂ laser spot. The ssDNA results shown 100,000-fold enrichment in less than 4 minutes. This preliminary results of aggregation

formation of streptavidin Q-dots and biotin labeled exosomes shown that this method can be used in biosample purification or quantification.

Also, using differential diffusivity of components in a fluid to condition liquid samples in a microfluidic device was presented. The method is particularly suitable for removing/adding components to adjust the ionic strength and buffer condition for downstream analysis as ions usually have higher diffusivity than bio-makers of interest such as exosomes, extracellular vesicles, proteins and DNAs. The removing components experiment results shown that nearly all fluorescein salt has diffused out of the chamber in 3mins while greater than 80% 40nm beads remain in the chamber. Most uniquely, the method does not require target capture and release during the process of changing the buffer, thus avoiding uncontrollable sample loss, contamination, and human intervention. The physical model accurately matches the experimental results and helps control the process and produce predictable results. Therefore, the method provides an attractive option to be incorporated into the workflow of biological sample preparation for biomedical tests. In addition, the method can be an effective means to measure the diffusivity of objects in the sample, which offers valuable insight for the physical properties of macromolecules such as their radius of hydration and geometry in different microenvironments.

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