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Single cell assay for analyzing single cell exosome and endocrine secretion and cancer markers

A dissertation submitted in partial satisfaction of the
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by

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2017

The Dissertation of Yu-Jui Chiu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2017

DEDICATION

To my family and loved ones…

EPIGRAPH

做好研究關鍵不在於你對於這個領域懂了多少，而在於

你能不能一眼看穿這個課題的本質與價值。

錢永健

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* These two authors contributed equally to this work

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ABSTRACT OF THE DISSERTATION

Single cell assay for analyzing single cell exosome and endocrine secretion and cancer markers

by

Yu-Jui Chiu

Doctor of Philosophy in Materials Science and Engineering

University of California, San Diego, 2017

Professor Yu-Hwa Lo, Chair

To understand the inhomogeneity of cells in biological systems, there is a growing demand for the capability to characterize the properties of individual single cells. Since single cell studies require continuous monitoring of the cell behaviors

instead of a snapshot test at a single time point, an effective single-cell assay that can support time lapsed studies in a high throughput manner is desired. Most currently available single-cell technologies cannot provide proper environments to sustain cell growth and cannot provide, for appropriate cell types, proliferation of single cells and convenient, non-invasive tests of single cell behaviors from molecular markers. In this dissertation, I present a highly versatile single-cell assay that can accommodate different cellular types, enable easy and efficient single cell loading and culturing, and be suitable for the study of effects of in-vitro environmental factors in combination with drug screening. The salient features of the assay are the non-invasive collection and surveying of single cell secretions at different time points and massively parallel translocation of single cells by user defined criteria, producing very high compatibility to the downstream process such as single cell qPCR and sequencing. Above all, the acquired information is quantitative — for example, one of the studies is measured by the number of exosomes each single cell secretes for a given time period. Therefore, our single-cell assay provides a convenient, low-cost, and enabling tool for quantitative, time lapsed studies of single cell properties.

Chapter 1

Introduction

1.1 Single Cell Analysis

Single-cell analysis provides information of individual cells that is often lost in measurements of large cell populations. Given the inhomogeneity of cells in biological systems, information from individual cells can be of critical importance in understanding biological processes and disease formation and progression such as variations in gene expression, drug resistance, and cancer metathesis. For instance, since 1970 it has been demonstrated the existence of distinct subpopulations of cancer cells within tumors, which differed in terms of tumorigenicity, resistance to treatment, and ability to metastasize[1–3]. Another example of the type of information that can be extracted from the inhomogeneity of cells is the subtly different neuronal cell types in the brain. Although it may be possible to detect those subpopulations in a bulk analysis, their signals tend to be masked by the whole population. And in any event, such analyses blur cell-to-cell distinctions, making it impossible to know which cells contribute what to the population[4]. The only way to untangle such important individual signals is to make measurements cell by cell: Analyzing single

cell behavior such as phenotypes and extracellular vesicles (EV) and endocrine secretion.

Extracellular vesicles (EV) such as exosomes (EX)/microvesicles secreted by tumor or normal cells were found to play important roles in cell-cell signaling[5], tumorigenesis[6], drug resistance[7,8], and organotropic metastasis[9]. Most studies in the biogenesis of EXs are performed over a cell population, in which the unique behaviors of minority or individual cells is masked. Exosome isolation[10] and characterization techniques, including advanced methods such as surface plasmon resonance and various microfluidic designs[11–15], are still unable to associate the properties of exosomes directly with their cell sources up to the resolution level of single cells.

1.2 Single Cell Isolation and Analysis Technique

1.2.1 Standard Single Cell Analysis Workflow

A standard technique to isolate single cell from a cell population is fluorescence-activated cell sorting (FACS) or a flow cytometer. A state-of-the-art flow cytometer can screen about millions of cells per second and then 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 (Figure 1.1). Although

this direct upstream (from the sample) to downstream (cell lysis) workflow is a reliable process, it lacks temporal information regarding cell behavior and cell-cell interaction such as proliferation ability, drug response, cell signaling, and so on.

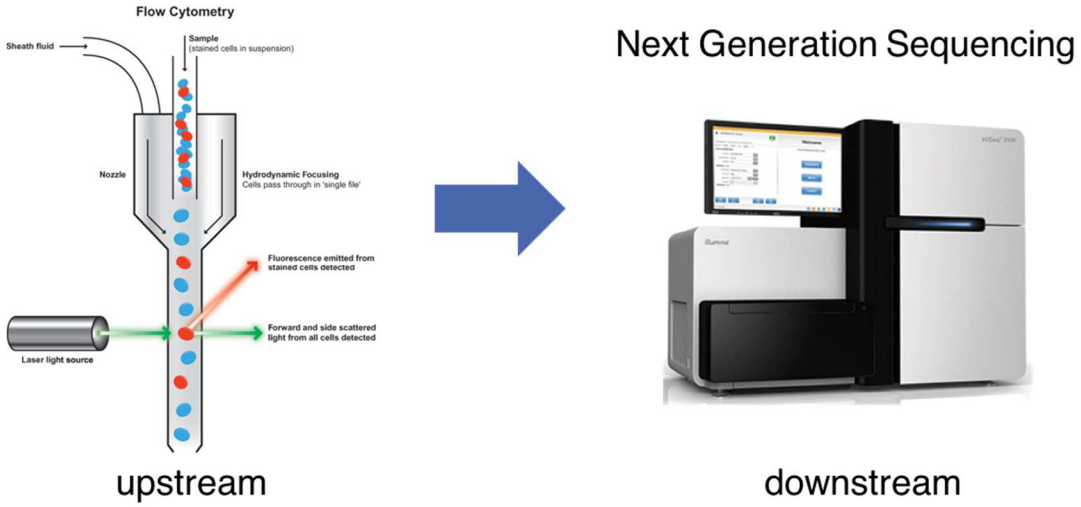


Figure 1.1: A Standard Single Cell Analysis Workflow

1.2.2 The Current Techniques

To fulfill the gap between the upstream process (FACS) and downstream process (gene analysis) several single-cell technologies have been developed and reported, including microfluidic devices[16–20], encapsulated droplet platforms[21,22], and printing methods[18,23]. Although these methods can capture single cells, most of them produce atypical environments for cell culture, which can decrease cell viability and alter cell behaviors, thus disrupting single cell studies. To address this issue, people have developed single-cell devices with surface modifications[24–28] to produce environments more compatible with conventional culture. However, these methods are cell specific and do not offer a general platform to support single cell studies of different cell types. Compared to closed systems that impose spatial confinements to cells, technologies that provide open systems allow easier control of cell culture environments (e.g. CO_2 level, oxygen level, nutrient and drug additions, etc.)[29–32].

1.3 Motivation

The main challenges have been on device fabrication and on operation such as cell placement and a high throughput single-cell assay that is versatile enough to support various downstream processes and analyses such as transfection, cell-cell interactions, time-lapse observations, and gene expression. It is also essential that the single-cell assay can achieve the downstream processes while maintaining good cell

viability, compatibility to standard bio-protocol, and even better flexibility for operation. A process offers the much needed solution for the process gap between FACS (Fluorescence-activated cell sorting) and single cell downstream analysis (e.g. qPCR, single-cell RNAseq, etc.).

1.4 Scope of Thesis

Here we present an open platform for parallel single cell analysis with the following salient features: (a) locating and tracking single cell behaviors as well as single cell secretions to enable correlation studies between phenotypes and secretion patterns, (b) allowing continual growth and development of single-cell derived micro colonies to support studies of single-cell genealogy and hereditary properties, and (c) enabling massively parallel translocation of single cells by user defined criteria. The combination of the above three capabilities plus the open platform (i.e. open to media change and modifications of microenvironments) offers enormous flexibilities and capabilities for single cell studies in high efficiency.

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

Chapter 3 will focus on a single cell platform by combining single cell culture chip and bio-printing technique into a single cell secretion quantification system.

In Chapter 4, a position-to-position parallel cell translocation technique will be introduced. The combinination of the single cell culture chip and bio-printing method, the platform is used for studying patient derived cancer cells.

Chapter 5 will summarize the dissertation and briefly discuss future work.

Chapter 2

Fabrication of single cell culture chip

In this chapter, we will discuss few novel methods for single cell culture chips; (1) PDMS micro through holes arrays using PDMS lift-off process, (2) A solvent free process for PDMS micro through holes arrays under ambient environment, (3) Combining PDMS arrays to cell culture dish, (4) A floating cell culture chip designed for both non-adherent and adherent cells.

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

The PDMS single cell loading mesh was made of PDMS lift-off (peel-off) process. A similar lift-off process had been reported by Park et al.[Cite] and Guo et al.[Cite]; however, these methods required precise process control and sophisticated skills. The fabrication process we developed here utilized a much simpler peel-off process with high tolerance and 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 bake for 1 minute. The patterns revealed after resist development. A 2D array of 100 μm deep, 40 μm diameter mesas were then etched by deep reactive ion etching (DRIE) process (Oxford Plasmalab 100) using the photoresist as etch mask (Fig. 2c). In the DRIE process, SF6 gas was flowed at 100 sccm for 11 s during the etching cycle, followed by a passivation cycle with C4F8 gas flowed at 80 sccm for 7 s. The etching rate was ~0.65 μm to ~0.7 μm per cycle and took about 150 cycles to etch ~100μm Si. The etched wafer was spin-coated by ~100 μm thick uncured PDMS (Sylgard 184, Dow Corning, MI) with a premixed 1:1 ratio (v/v) with hexane 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 12mm inner diameter and 14mm 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.

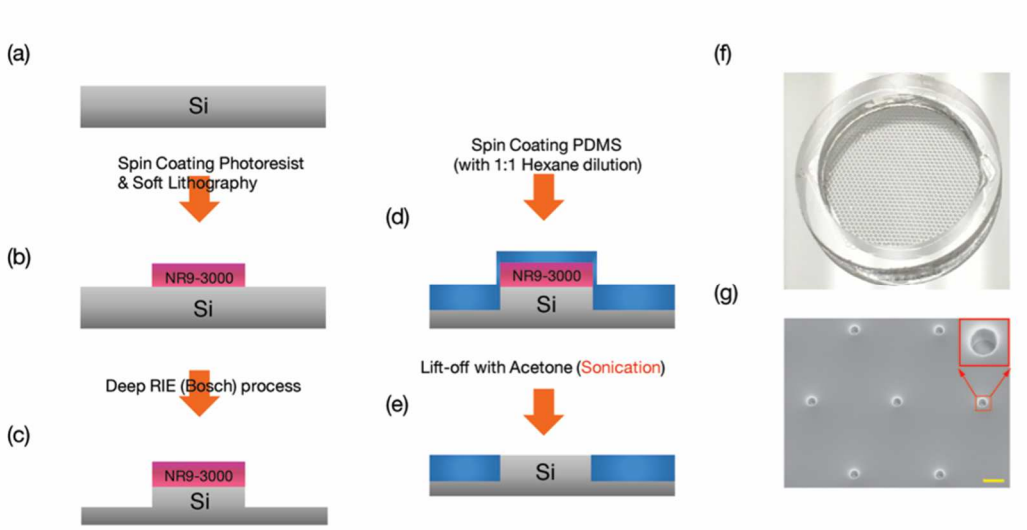


Figure 2.1: Process flow for fabrication of PDMS mesh to aid cell loading. a) Prepare a clean Si-wafer. b) Form 6 µm thick photoresist NR9-3000 PY patterns. c) Using photoresist as mask for deep RIE process to form 100 µm deep, 40 µm diameter mesas. d) Spin coat sylgard 184 with 1:1 hexane dilution e) 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 micrograph of the PDMS mesh with a 2D array of through holes to aid cell loading. The red square shows the closer view of one hole. The scale bar is 100 µm.

2.2 Combining PDMS arrays to cell culture dish

The overall work flow of the single cell assay is shown in Figure 2.2. We first fabricated a polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, MI) mesh with a two-dimensional array of through holes (40 μm diameter) using direct lithographic lift-off of the PDMS layer, as shown in Figure 2.2(a) and in Figure 2.1 for the detailed process flow. This PDMS mesh was tentatively adhered to a glass substrate 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.

The PDMS mesh was treated with oxygen plasma (100 Walt for 30 seconds) to provide enough bonding strengths with the glass plate to sustain cell loading, but the bonding could be separated by mechanical shear after cell loading. After filling the wells with the culture medium (Figure 2.2(a)), 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 glass. Then one can gently remove the PDMS mesh from the glass substrate without disturbing the cells. The process flow is illustrated in **Figure 2.2**.

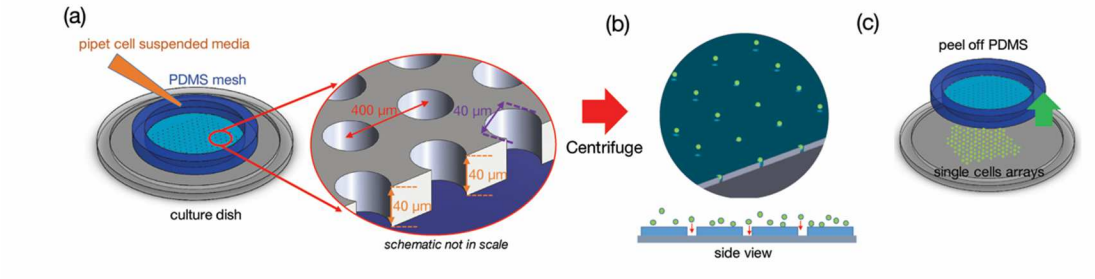


Figure 2.2: Single-cell assay used for analyzing exosome secretion. a) Loading single cells onto a culture plate utilizing a PDMS mesh which can be removed afterward. b) The mesh was removed after cell attachment. c) A surface functionalized glass slide was placed on the support frame 100 µm above the cells.

2.3 A cell culture chip designed for both non-adherent and adherent cells

Given that in every experiment the locations of cells of interest are unpredictable, the cell culture chip cannot be mass produced but be individually generated by users rather than by chip vendors. To meet this requirement, a solvent free fabrication process of single cell culture chip has been developed. At first, print the UV exposure mask on a transparency using an office-grade laser printer. Coated UV-patternable PDMS (UV-PDMS) on a piece of cover glass and placed it on top of the transparency mask. Exposed the UV-PDMS through the mask by a UV lamp (100 W, about 0.5 W/cm2) for 20 seconds. The UV-cured PDMS was removed from the cover glass by sonication in a water bath. After being dried in air, the PDMS surface was treated with UV-Ozone (or oxygen plasma) before being bonded to the polyester membrane filter. Before cell transfer or loading, the chip was UV-Ozone (or oxygen plasma) treated again to enhance hydrophilicity.

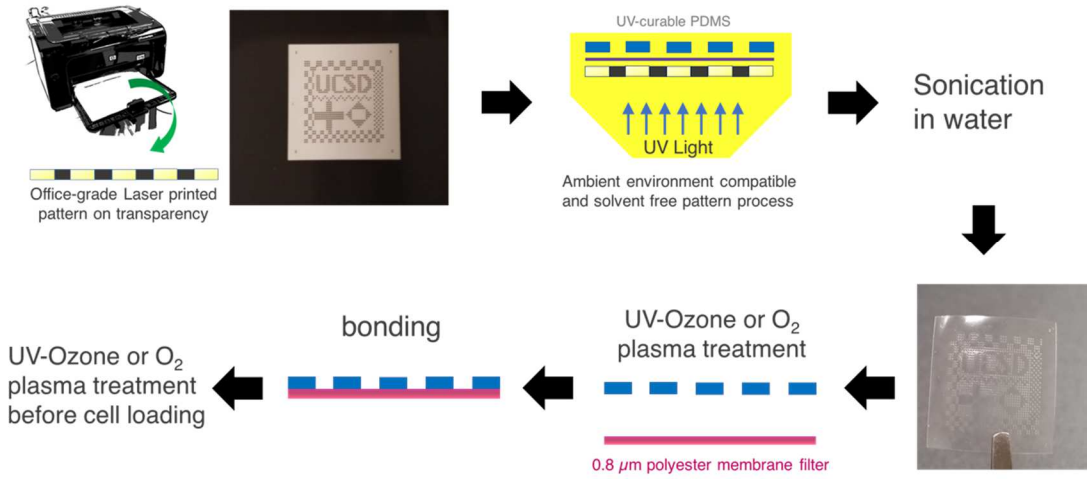


Figure 2.3: Fabrication of single cell culture chip for both adherent and non-adherent cells.

This chapter is based on and mostly a reprint of 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 Yu-Jui Chiu, Wei Cai, Tiffany Lee, Julia Kraimer and Yu-Hwa Lo. (2017). Quantitative Analysis of Exosome Secretion Rates of Single Cells. Bio-protocol 7(4): e2143. The dissertation author was the primary investigator and author of this paper.

Chapter 3

A Single Cell Assay for Studying Single Cell Secrete Exosomes Using a Bio-printing Method

3.1 Overview

A single-cell assay that is applicable to different cell types as a platform technology and offers the throughput, versatility, and precision required for quantitative single-cell investigations. The device was fabricated by direct lithographic patterning of polydimethylsiloxane (PDMS) material into a mesh to guide cell loading with high throughput and accurate positioning. Subsequent cell culturing and time-lapsed studies were performed in a natural culture environment that allows for harvest of single-cell secretions noninvasively. To demonstrate the unique features as a single-cell assay, we have used the technology to quantify the rate of exosome secretion by single cells over a period of 24–96 h. Such studies are of biological significance but have never been conducted before due to lack of proper technologies.

3.2 Workflow from Cell loading to Exosome Collection

The overall work flow of the single cell assay is shown in **Figure 3.1**. We first fabricated a polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, MI) mesh with a two-dimensional array of through holes (40 μm diameter) using direct lithographic lift-off of the PDMS layer, as shown in Fig. 1(a) and in Fig. 8 for the detailed process flow. This PDMS mesh was tentatively adhered to a glass substrate 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.

The PDMS mesh was treated with oxygen plasma (100 Walt for 30 seconds) to provide enough bonding strengths with the glass plate to sustain cell loading, but the bonding could be separated by mechanical shear after cell loading. After filling the wells with the culture medium (Fig. 1(a)), 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 glass. Then one can gently remove the PDMS mesh from the glass substrate without disturbing the cells. The process flow is illustrated in Figure 1.

We subsequently applied a bioprinting method to assay single cells from the position registered single cell array. To collect secretions such as cytokines or

vesicles by each individual cells regularly without disturbing the cells, a surface functionalized collection glass slide was placed atop the cells (with a separation of 0.1 mm from the cell surface) without physically touching them. The process design for collection of single cell secretions is illustrated in Fig. 1 (d)-(f). A 2 mm thick 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 (Qdots), as shown in Figure 1(f). Using an inverted fluorescent microscope to count quantum 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, we investigated proteins CD63, CD9, and CD81 on the surface of exosomes. As a platform technology, the method can be used to collect any single cell secretions with specifically treated collection glass slides.

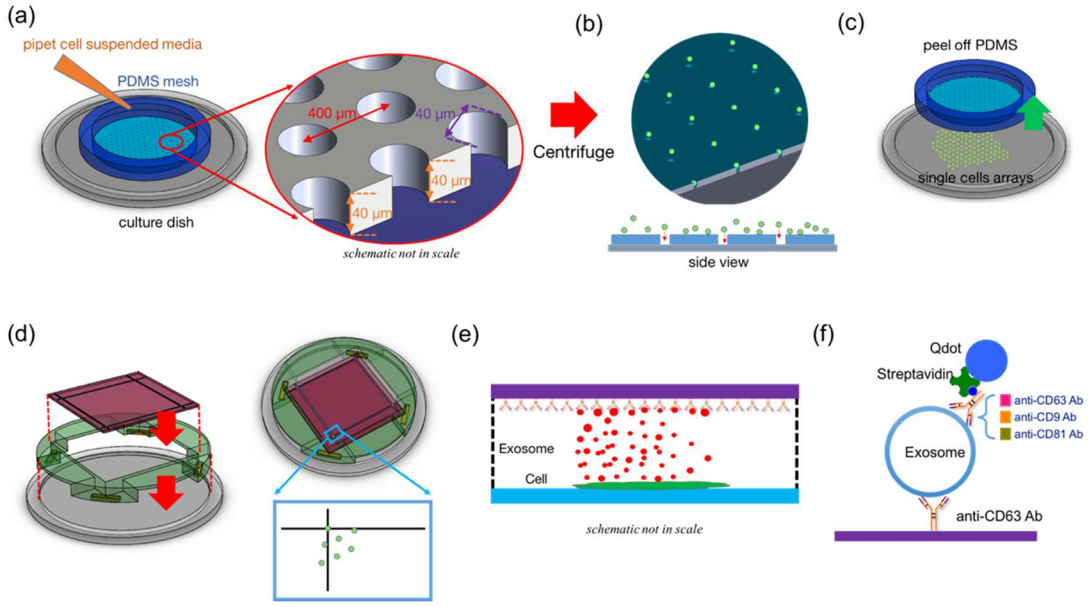


Figure 3.1: Single-cell assay used for analyzing exosome secretion. (a) Loading single cells onto a culture plate utilizing a PDMS mesh which can be removed afterward. (b) The mesh was removed after cell attachment. (c) A surface functionalized glass slide was placed on the support frame 100 µm above the cells. (d) and (e) 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 and single cell loading

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% CO_2. 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.

3.4 Antibody immobilization on cover glass

To immobilize anti-CD63 antibody on the cover slide for exosome capture, we diced and silanized the cover glass in 4% v/v (3-mercaptopropyl) trimethoxysilane (MPS) for 30mins with 200rpm spin speed at room temperature. The silanized cover slide was then washed three times with ethanol, followed by 105^{o}C heating for 30 mins until it fully dried. The cover slide was incubated in 0.1 mM cross-linker, in 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 ^{o}C for 2 hours, follower by PBS wash for 3 times. The cover slide was then immersed in PBS with 5% (w/v) bovine serum albumin (BSA) at 4^{o}C for 30 minutes to passivate non-reacted Sulfo-GMBS. After PBS wash for 3 times, the functionalized slide was stored in PBS at 4^{o}C and ready to use.

3.5 Imaging and Counting

After exosomes were collected to the functionalized cover slide, the cover slide 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 ^{o}C for 2 hours, and washed with PBS for 3 times again. Then 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 Qdots (Life Technologies) solution at room temperature for 1 hour, followed by three times of 50^{o}C TBST wash and 2 times of 50^{o}C deionized (DI) water wash before dehydration by series immersion of the slide in diluted ethanol with DI water. 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 Qdots were counted by using an object counter module in the microscope software (BZ-II Analyzer).

3.6 Characterization of the platform

After cell loading, we waited 4 to 6 hours before removing the PDMS mesh from the cell culture plate to give the cells enough time to anchor on the glass surface. The 2D array of cells was cultured and regularly observed under microscope to monitor cell viability. Fig. 2(a) shows the image of GFP transfected MCF7 cells under 4X objective lens (BE-II 9000, Keyence) after 6 hours since 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. Figure 2 (a) shows the experimental results using 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. In this experiment, single cells were cultured up to 96 hours for time lapsed studies, showing that the technology and process can maintain cell viability at single cell levels with the closest neighboring cells being 400 μm apart.

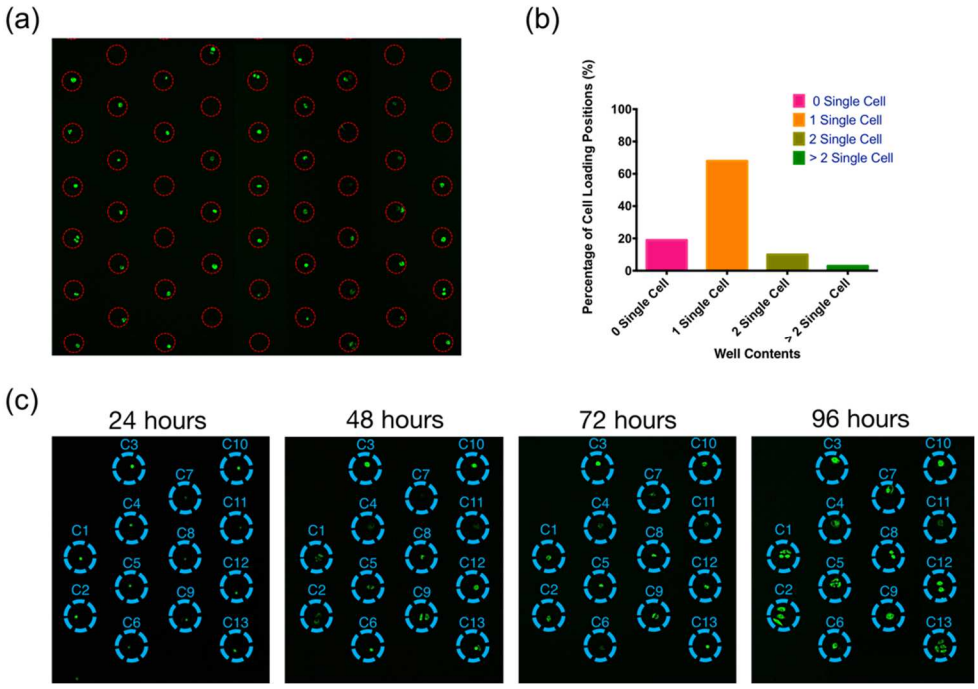


Figure 3.2: Cell Loading and culturing. (a) Distribution of GFP transfected MDA-MB-231 cells. (b) Statistic distribution of cell numbers. (c) Time-lapsed observations of single cell culture. All cells survived and proliferated.

The maximum exosome capturing capacity of the glass slide, immobilized with anti-CD63 Ab, was tested with samples containing a high concentration (~2x104/μL) of exosomes in phosphate buffered saline (PBS) solution. It was determined that within the 110 μm x 150 μm microscopic field of view under 100X objective lens, a maximum number of ~1500 anti-CD63 Ab conjugated Quantum dots (Qdots) can be bonded to the surface, setting the upper limit of detectable density of exosomes. Therefore, in this study we have kept the exosome collection time to 3 hours so that the highest number of Qdots 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, we have analyzed exosome counts over areas without corresponding cells but with cells in neighboring areas. As shown in Figure 3, the exosome counts in those cell-absent areas were comparable with the counts in the background level due to non-specific binding of QDs, about 25 over the field of view. The results confirmed that about 3% of signal might come from crosstalk caused by exosome diffusion.

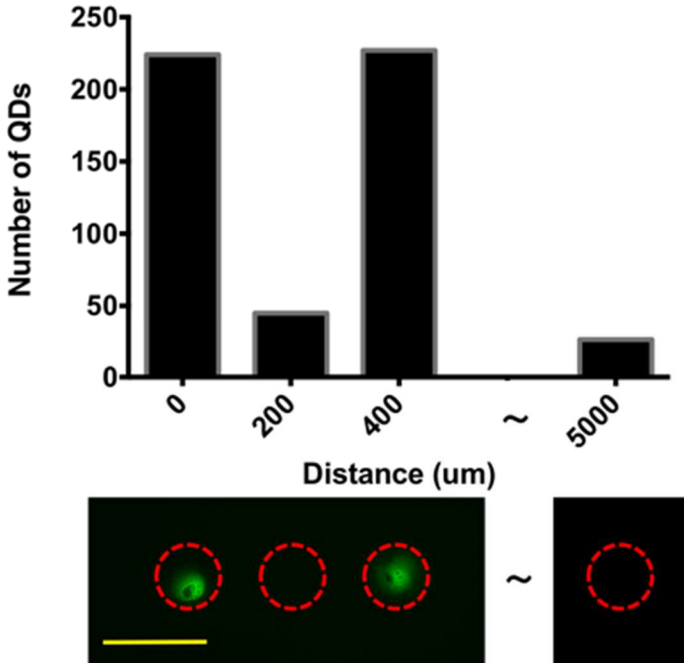


Figure 3.3: Evaluation of crosstalk due to exosomes secreted by the neighboring sites. The result shows that the crosstalk is slightly above the background level and contributes to about 3% of the signal. The scale bar in the lower picture represents 200um.

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 singe cell from the corresponding position. The collection time was chosen to be 3 hours for each measurement, and each experiment typically lasted 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 3.4**, 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. Also for those exosomes produced by MCF10A cells and captured by the anti-CD63 Ab probes immobilized to the glass slide, 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[33,34].

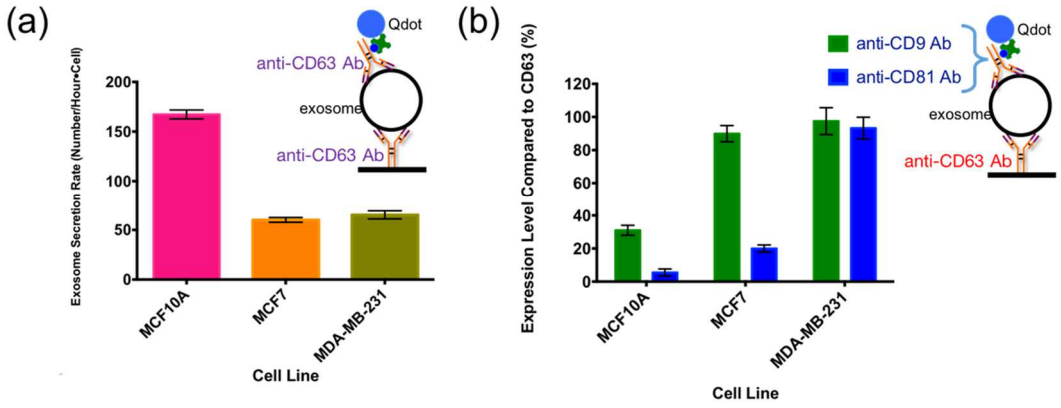


Figure 3.4: Expression level of different surface marker on different cell lines. (a) Exosome secretion rate (in number/hour-cell) characterized by CD63 protein labeled with biotinylated anti-CD63 Ab and Qdots. (b) The percentage of CD9 and CD81 positive exosomes among all the exosomes captured by anti-CD63 antibody.

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

We further demonstrate that, as a 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 doses[35]; 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 2 mils of exosome-free culture medium to achieve a final concentration of 5 ng/mL. After adding the drug containing medium into the culture dish, CD-63 positive exosomes were collected for 3 hours using the anti-CD63 Ab immobilized glass. After exosome collection, the cells were imaged and the quantum-dot labelled exosomes at the corresponding positions to the cells were recorded by using a fluorescent microscope. Every 24 hours from the last collection, the old medium was aspirated and replaced by 2 mils of new exosome-free medium with 5ng/mL paclitaxel. The above steps were repeated throughput the 96 hours of study period.

Figure 3.5 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 ~20% after 24 hours. These results provide more quantitative information to support that MDA-MB-231 may have higher chemoresistance than MCF7[36] so was less responsive to paclitaxel. To further quantify the effect of the drug on the expression level of CD9 and CD81 among the CD63 positive exosomes, we define the impact factor as Equation (1).

$$F_{CD9} = \left(\frac{R^*_{CD9}}{R^*_{CD63}} - \frac{R_{CD9}}{R_{CD63}}\right)/\left(\frac{R_{CD9}}{R_{CD63}}\right) \text{ and } F_{CD81} = \left(\frac{R^*_{CD81}}{R^*_{CD63}} - \frac{R_{CD81}}{R_{CD63}}\right)/\left(\frac{R_{CD81}}{R_{CD63}}\right) \quad (1)$$

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. F>0 and F<0 means the perturbation introduces positive and negative (i.e. increasing / decreasing the expression level of specific protein in exosomes). The inserts in Fig. 5 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.

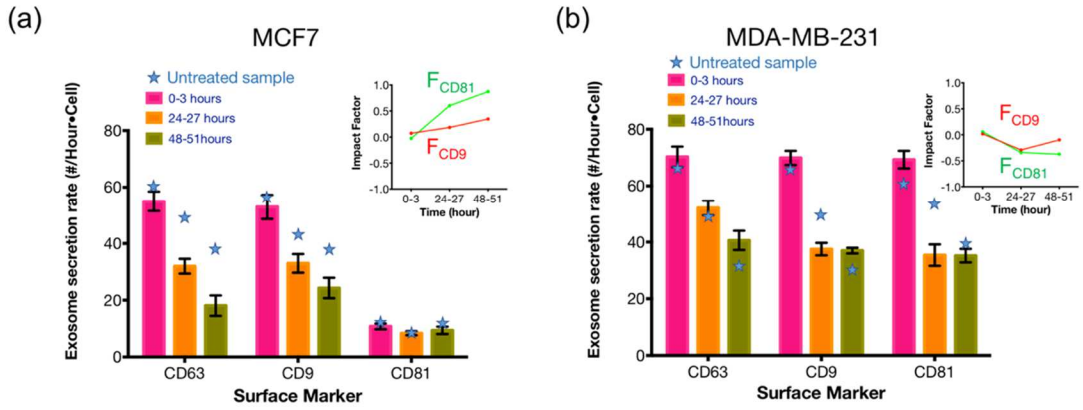


Figure 3.5: Expression level of different surface markers after paclitaxel treatments for (a) MCF7 and (b) MDA-MB-231. The star symbols indicate the secretion rate without paclitaxel treatment. The main figures show the exosome secretion rates (in number/hour-cell) for CD63 positive exosomes that are 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.

There have been studies that cell's exosome release may be affected in lower than physiological pH value[37,38]. We have applied the technology platform to conduct a more quantitative study of exosome secretion in an acidic environment. Similar to the paclitaxel test, the cells were cultured for 48 hours before adding 2 mils of 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, cells were imaged and the collected Qdot-labeled exosomes were recorded by using a 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.

We used the same way as we did in drug tests to evaluate the exosome secretion and the impact factor due to the pH change. As shown in **Figure 3.6**, 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 those 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 a transient instead of a permanent effect since in 24-27 hours, the expression levels of CD9 and CD81 recovered to almost the same level as they were in regular pH medium. On the other hand, the expression levels of CD9 and CD81 for MDA-MB-231 cells 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.

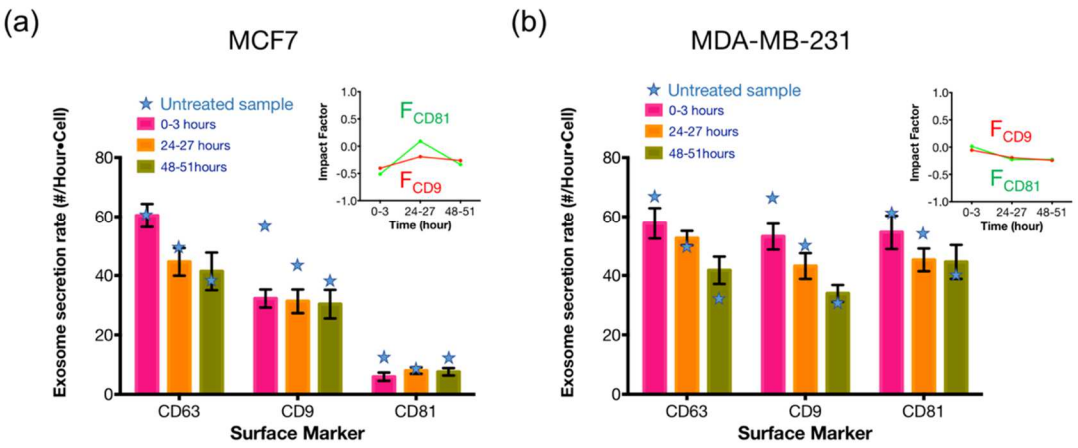


Figure 3.6: Expression levels of different surface markers after being cultured in lower pH (6.7) media for (a) MCF7 and (b) MDA-MB-231. The star symbols indicate the secretion rate in normal pH (pH=7.4) medium. The main figures show the exosome secretion rates over time for CD63 positive exosomes that 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, we also investigated how cells responded to certain proteins such as transforming growth factor beta (TGF-β), a protein that was reported to induce MCF7 cell epithelial-to-mesenchymal transition. In our 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 3.7**, 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 very significantly (with impact factor reaching +2) while the response of CD9 to TGF-β was modest.

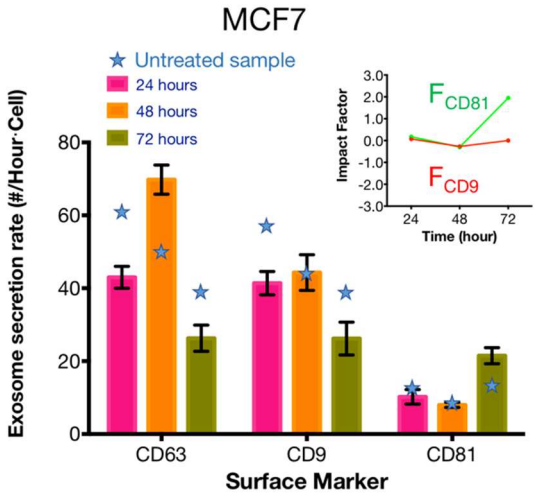


Figure 3.7: Expression level of different surface markers after the TGF-β treatment for MCF7. The star symbols indicate the secretion rate without TGF-β treatment. The main figure shows the exosome secretion rates over time for CD63 positive exosomes that are simultaneously CD9 and CD8 positive. The insert shows the impact factor of the perturbation on MCF7.

3.9 Summary

In summary, we have presented a single-cell assay that is applicable to multiple cell types, friendly for cell loading and operation, supportive for time-lapsed studies, and capable of non-invasive and quantitative analysis of molecular markers and vesicles secreted by single cells. The device is simple, low cost, versatile, and with moderately high throughput to support most single-cell studies. Notably, this platform has demonstrated to support excellent single cell viability with continuous data up to 96 hours. Specifically, we have shown the capability of the platform to quantify the various exosome secretion rates of single cells under different in-vitro conditions. In these studies, CD63 positive exosomes secreted by single cells in a 2D array were captured regularly, enabling measurements of exosome secretion rates by individual cells. Two additional surface markers, CD9 and CD81, were used to study the exosomal expression levels for cells under different external perturbations such as drug treatments, pH changes, and growth factors. While the main focus of these studies was to demonstrate the capabilities and versatility of the single-cell assay, the results of studies, including the effects of paclitaxel treatment, acidity, and TGF-β on MCF7 and MDA-MB-231 breast cancer cells, have provided valuable biological insight by the quantitative data at single cell resolution.

This chapter is based on and mostly a reprint of 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 Yu-Jui Chiu, Wei Cai, Tiffany Lee, Julia Kraimer and Yu-Hwa Lo. (2017). Quantitative Analysis of Exosome Secretion Rates of Single Cells. Bio-protocol 7(4): e2143. The dissertation author was the primary investigator and author of this paper.

Chapter 4

An Open Platform for Single-Cell Translocation and Secretion Assay

4.1 Overview

Most studies in the biogenesis of extracellular vesicles (EV) such as exosomes are performed over a cell population, in which the unique behaviors of minority or individual cells are masked[9,12,39,40]. Here we present an open platform for parallel single cell analysis with the following salient features: (a) locating and tracking single cell behaviors as well as single cell secretions to enable correlation studies between phenotypes and secretion patterns, (b) allowing continual growth and development of single-cell derived micro colonies to support studies of single-cell genealogy and hereditary properties, and (c) enabling massively parallel translocation of single cells by user defined criteria. The combination of the above three capabilities plus the open platform (i.e. open to media change and modifications of microenvironments) offer enormous flexibilities and capabilities for single cell studies in high efficiency. In this paper, we present the core technology of the open platform single-cell assay and demonstrate its unique capabilities with

exemplary studies of single-cell exosome secretion rate dependence on cell life cycles and genealogy.

4.2 The TransSeA Technology

The open platform of the single-cell translocation and secretion assay (TransSeA) has three technology components: templates for single cell culture[41,42], single cell secretion harvesting, and parallel translocation of targeted cells. The assay provides an enabling tool to link individual cell behaviors, especially behaviors of rare cells, and single-cell genomics in a highly efficient manner.

4.2.1 Fabrication of single cell culture plate

The first part of TransSeA is a single cell culture chip (Figure 4.1) consisting of a polyester thin film filter attached to a layer of PDMS through-holes[41]. The polyester filter provides the substrate for cell attachment and the PDMS through-holes provides physical confinements and position registration of individual cells. The pore size of polyester thin film filter (e.g. 0.8μm) is chosen to allow passing of cell secretions while supporting the cells. At first, print the UV exposure mask on a transparency using an office-grade laser printer. Coated UV-patternable PDMS (UV-PDMS) on a piece of cover glass and placed it on top of the transparency mask. Exposed the UV-PDMS through the mask by a UV lamp (100 W, about 0.5 W/cm2) for 20 seconds. The UV-cured PDMS was removed from the cover glass by

sonication in a water bath. After being dried in air, the PDMS surface was treated with UV-Ozone (or oxygen plasma) before being bonded to the polyester membrane filter. Before cell transfer or loading, the chip was UV-Ozone (or oxygen plasma) treated again to enhance hydrophilicity. After oxygen plasma treatment, the single cell culture chip was assembled into a CNC (Computer Numerical Control) machined fixture.

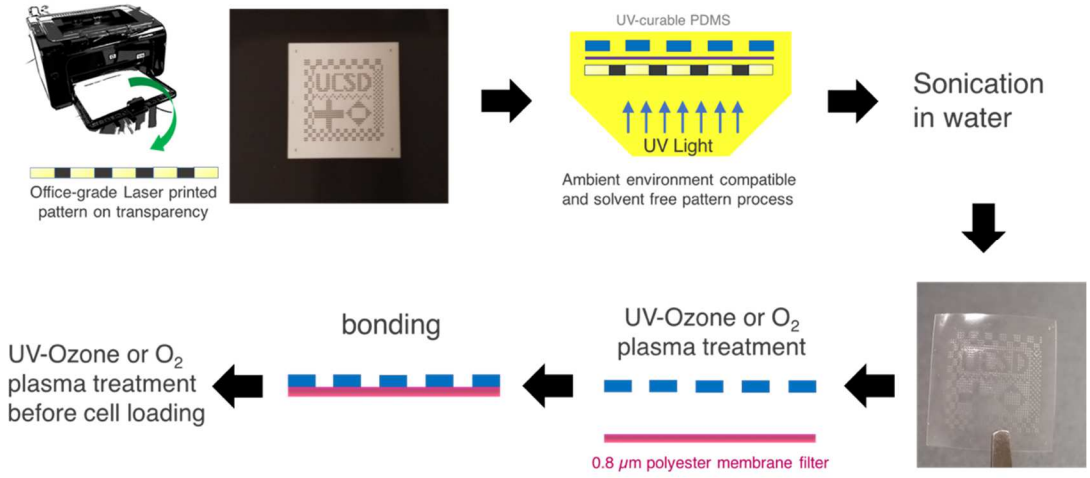


Figure 4.1: Fabrication of single cell culture chip

4.3 Single cell secretion harvesting and profiling

For periodic harvesting of cell secretions, an antibody functionized secretion collection plate[41,42] was placed atop the cell culture fixture with a well-defined and adjustable spacing (typically 100μm to 250μm). Alternatively, if applications prefer collection of secretions in suspension rather than being immobilized on a glass substrate, secretions can be drained into wells underneath the cell culture fixture via the pores of the filter. In this paper, we study exosome (EX) secretion rate as an example for quantitative single cell analysis with TransSeA. Exosomes secreted by individual GFP-labelled GBM3 cells (a short-term passaged, patient derived glioblastoma line) were enumerated. The results from 193 single GBM3 cells are summarized in Figure 1 B where the EX secretion rate is binned with an increment of 10. The data show extraordinarily large inhomogeneity in exosome secretion rate among individual cells, ranging from 2 EX to 218 EX in 3 hours 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 EX per 3 hours and another with an average secretion rate of 115 EX per 3 hours (**Figure 4.2**)

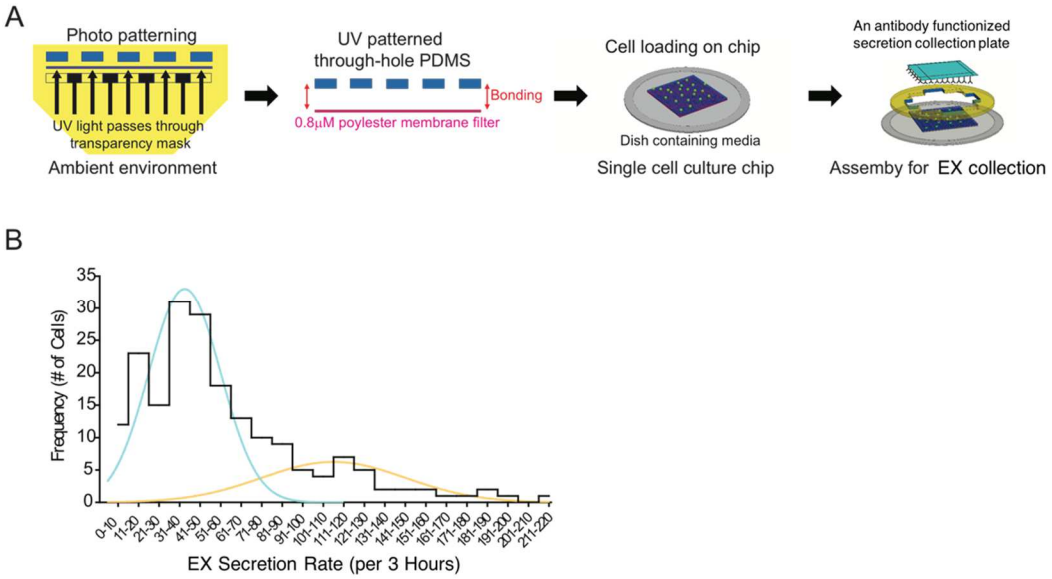


Figure 4.2: Single cell culture and single cell secretion harvesting of TransSeA. (A) Single cell culture chip consisting of a polyester thin film filter attached to a layer of PDMS through-holes. An antibody functionized secretion collection plate is placed for single cell secretion harvesting. A CNC machined fixture is assembled for confining the collection plate. (B) Summary of EX secretion rate from 193 single cells. The data show extraordinarily large inhomogeneity in exosome secretion rate among individual cells, and are best fitted by two distribution curves, one with an average secretion rate of around 40 EX per 3 hours and another with an average secretion rate of 115 EX per 3 hours.

4.4 Will Exosome secretion rate be cell cycle dependent?

The TransSeA technique enables us to study single cell behaviors quantitatively and with high temporary resolution. One example of such studies is to find whether and to which extent the exosome secretion rate of a cell depends on its life cycle. Since it is not easy to identify the exact stage of life cycle for a cell without disturbing it, we designed the experiment by measuring exosome secretion rate of 41 randomly chosen single GBM3 cells in 6 hours interval (i.e. first 3-hour collection, 3 hours wait before the second 3-hour collection). Since mammalian cells have a full cell cycle of 20-24 hours with each state in the cycle lasting for 1 to 10 hours (**Figure 4.3** A), the sample size (41 single cells) in the experimental design covers essentially all possible situations for cells to transit from one state to another. Should the exosome secretion rate be cell cycle dependent, then the first and second exosome secretion rate measurements from the same cell should have high a probability to show appreciable difference since the first and second exosome collection likely occurs in different cell cycles. Conversely, should the exosome secretion rate be cell cycle independent, then the difference between two measurements is expected to be minimum for all cells being studied. To remove any bias between the two measurements, we normalize the value of each measurement to the mean value of all 41 cells. The above analysis process can be represented mathematically by Equation 1:

$$R = \left| \frac{\left(\frac{1st\ secretion}{Avg1}\right) - \left(\frac{2nd\ secretion}{Avg2}\right)}{\left(\frac{1st\ secretion}{Avg1}\right) + \left(\frac{2nd\ secretion}{Avg2}\right)} \right| \qquad (1)$$

Avg1 and Avg2 represent the average secretion rate of the first and the second EX collection over the entire population of 41 cells (**Figure 4.4**). The value of R in Eq. (1) is between 0 and 1, being a measure of the level of exosome secretion rate dependence on cell cycle. R approaches 0 if there is no dependence on cell cycle and R approaches 1 if the exosome secretion rate changes greatly at different stages of cell cycle. **Figure 4.3** B shows that for 40 out of 41 cells, the R value was between 0.1 and 0.2, and only 1 cell had its R Value of about 0.3. The results suggest that for all cells being studied, the exosome secretion rate changes little among different stages of cell cycle. Therefore, when we investigate exosome secretion rate variations among different cells, the stage of life cycle for the cell would not affect the result.

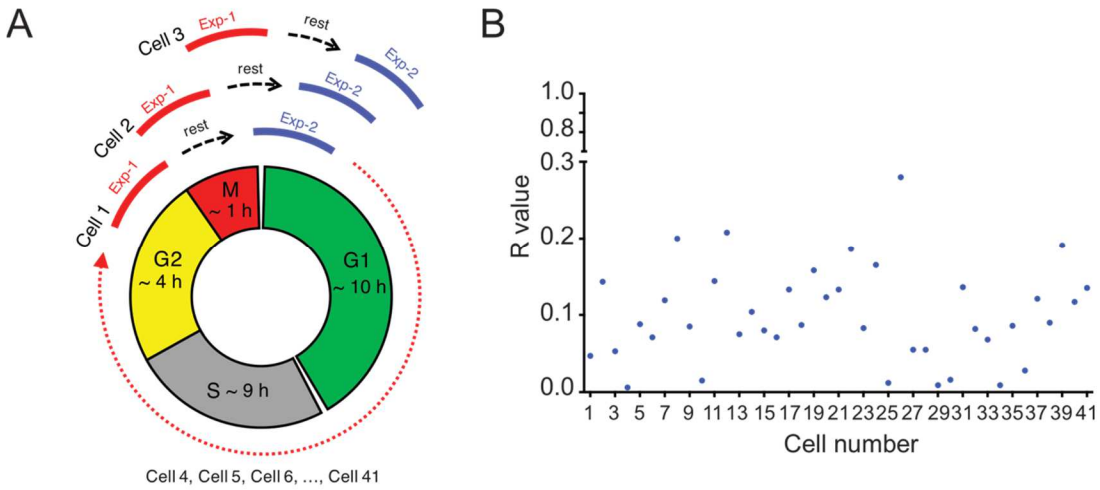


Figure 4.3: Exosome secretion rate and cell cycle dependence. (A) Experimental design for exosome secretion rate and cell cycle dependence. Measuring exosome secretion rate of randomly chosen single GBM3 cells in 6 hours interval (i.e. Exp1 refers to first 3-hour collection, 3 hours wait before the Exp2, the second 3-hour collection). (B) Summary of the results. 40 out of 41 cells has R value in between 0 to 0.2 and only one is around 0.3. For all cells being studied, the exosome secretion rate changes little among different cell cycle.

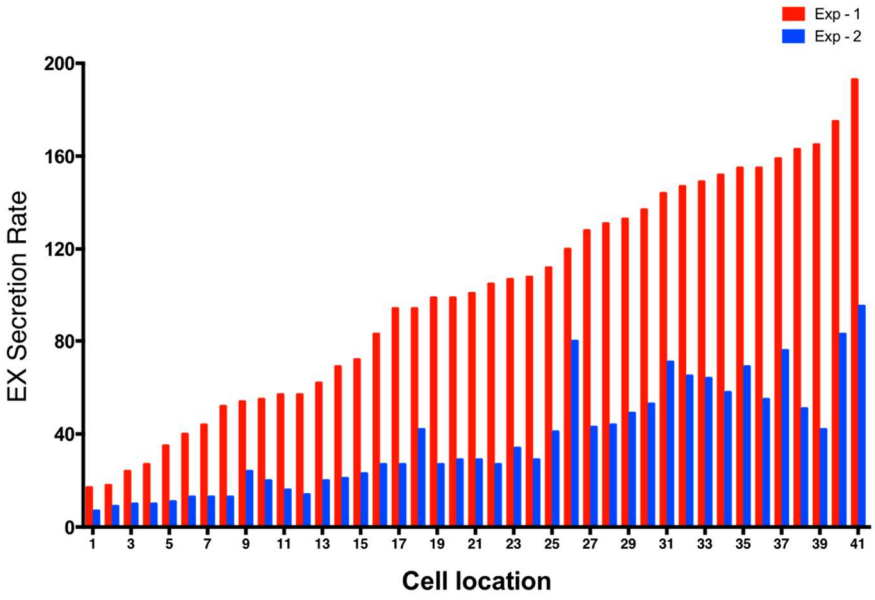


Figure 4.4: Exosome secretion rate of single GFP-labelled GBM3. Exosome secretion rate of single GFP-labelled GBM3. The red bars are exosome secretion rates over the first three-hour collection time. The blue bars are the exosome secretion rates over the second three-hour collection time. There is a three-hour rest period between the first and second three-hour collection.

4.5 Target cell selection and massively parallel cell transfer

One key feature of single-cell TransSeA is to allow users to select individual cells of interest according to phenotype, antibody labeling, FISH labeling, secretion properties[43–45], etc. and isolate and transfer them in a massively parallel manner to a new template that tracks the position and identity of individual cells without external barcodes.

To demonstrate such capabilities, here we use cell EX secretion rate as the criterion for cell selection. After quantification of single cell exosome secretion rate from harvested exosomes on a glass substrate, we cultured cells for 3 days to produce single-cell derived micro colonies consisting of 2 to 6 cells for each colony. According to the EX secretion rate of the parent cell, we selected specific single-cell derived colonies for further studies using the technique of parallel cell translocation, illustrated in **Figure 4.5** (A,B).

The chip that receives the transferred cells has a similar structure as the original cell plate discussed above with two additional features. At first, while the original cell plate can be prefabricated in the microfabrication cleanroom with predetermined, periodic PDMS through-holes attached to a filter substrate, the receiving template has user defined patterns based on user defined cell selection criteria. Secondly, given that in every experiment the locations of cells of interest are unpredictable, the cell receiving plate cannot be mass produced but be individually generated by users rather than by chip vendors. To meet this

requirement, for TransSeA we make the cell receiving plate out of UV-sensitive PDMS with the UV mask pattern produced by an office-grade laser printer. The UV-patterned PDMS through-holes require no mode master as in conventional soft lithography process, and can be fabricated in biological labs without sophisticated tools or dedicated microfabrication facility. **Figure 4.5** (bottom) shows an example of parallel cell transfer where four micro colonies of GFP-labelled GBM3 cells were transferred in parallel to four corresponding arrays of single cells.

4.5.1 Will Exosome secretion rate hereditary through cell genealogy?

One unanswered question that could have significant ramifications in fundamental and clinical biomedicine is whether and to what extent EX secretion of a cell is hereditary. In other words, will the high (or low) exosome secretion of a cell pass on to its direct descents?

The TransSeA technique is employed to answer the above question, as illustrated in **Figure 4.5** A. The above mentioned design of user defined parallel cell translocation was adopted with an additional twist of redistributing cells from each microcolony. The latter process separates cells from each single-cell derived microcolony (typically containing 2-6 cells for each colony representing 1-3 generations of cell division) into individual single cells onto a new template (**Figure 4.5** B). We studied those individual cells with their EX secretion rate significantly

higher or lower than the mean value. For cell translocation, an array of PDMS through-holes was formed at the positions of selected cells by using the aforementioned UV-patternable PDMS process. We then added another layer of small (80 μm in diameter) through-holes (**Figure 4.5** B) in contact with the cell receiving polyester filter in the sandwiched structure of the cell translocation assembly. The purpose of the sandwiched layer of small through-holes is to spread the cells from one microcolony into individual locations. The microfluid flow that drove the chosen cells from their original template to the receiving template was produced by applying a vacuum (~0.7 atm) at the cell receiving end to suck the liquid (1X PBS) atop the cell transmitting end. The CNC machined cell transfer assembly provides good sealing and uniform pressure over the template area for high transfer yield and low cell loss (<10%). **Figure 4.5** D shows the exosome secretion rate from 14 parental cells (10 low and 4 high EX secretion rate) and their descendants. According to **Figure 4.5** C, we classified cells secreting less than 60 exosomes in 3 hours as Low EX (blue) and cells secreting more than 80 exosomes in 3 hours as High EX (red). Those cells that secrete 61 to 80 exosomes in 3 hours were considered to have intermediate secretion rate (grey). The results in **Figure 4.5** E indicate that there is about 20% chance for Low EX cells to produce High EX decedents. On the other hand, there exists a much greater chance (~75%) for High EX cells to produce Low EX decedents. The study seems to suggest that the EX secretion rate does *not* appear to be hereditary and low EX rate appears to be the "norm" for GBM3 cells (most High EX cells produced low EX descents) while some

low EX cells can occasionally (around 20% chance) produce high EX descents. Although the results are too preliminary to be conclusive from biology standpoints, the study demonstrates the unique capability of the TransSeA as an enabling technique to investigate key unanswered questions in biomedicine.

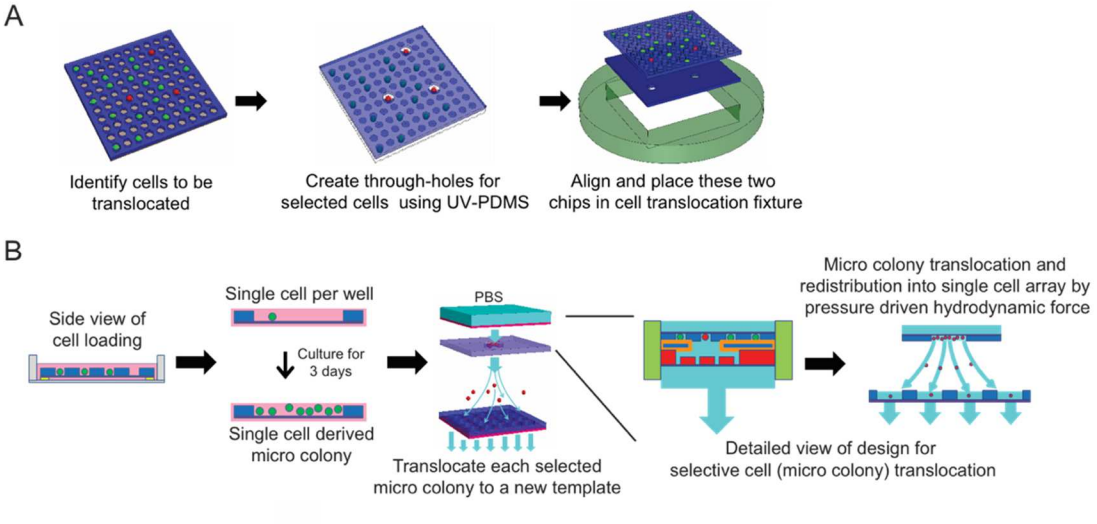
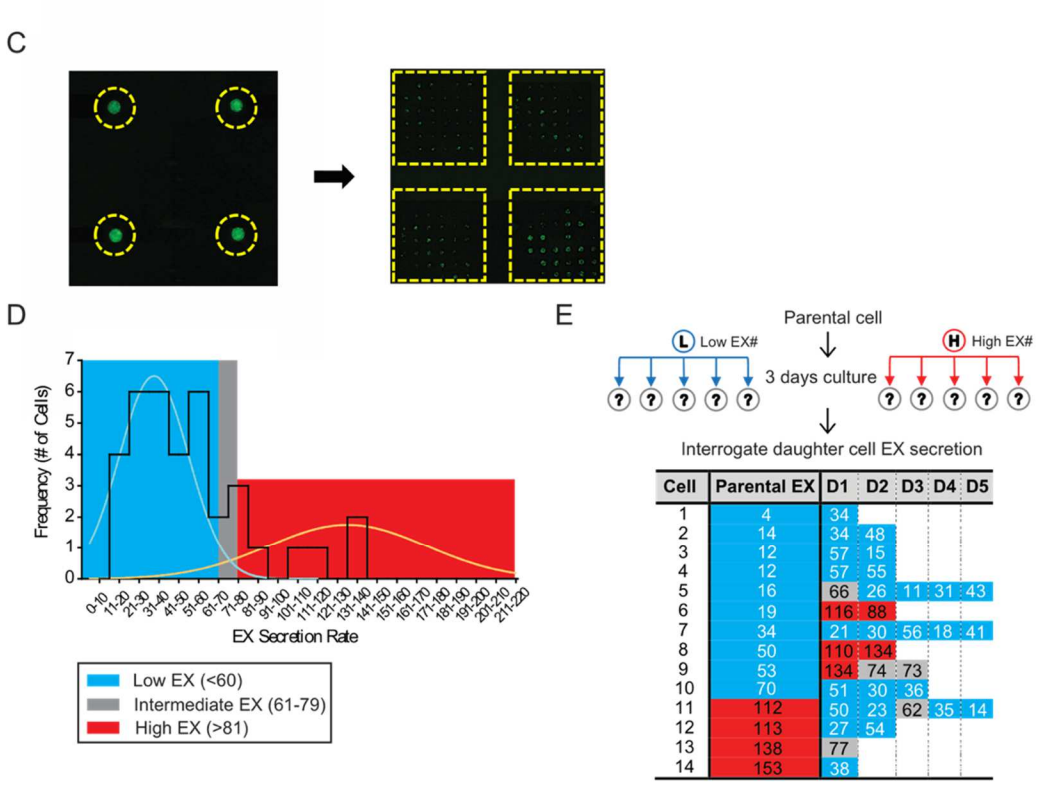


Figure 4.5: Parallel cell transfer and study of exosome secretion rate heredity through cell genealogy. (A) The positions of targeted cells on the cell culture chip, marked red, were defined by a layer of UV-patterned PDMS through-holes. The chip stack was flipped and assembled in a cell translocation fixture. (B) According to the patterns in (A), cells in each chosen micro colony was translocated to a new template consisting of arrays of 80 μm diameter through-holes in contact with the cell receiving polyester filter. During translocation, the cells in each micro colony were spread to individual locations in the array due to the pressure (~0.7 atm) driven microfluidic flow.



Cell	Parental EX	D1	D2	D3	D4	D5
1	4	34				
2	14	34	48			
3	12	57	15			
4	12	57	55			
5	16	66	26	11	31	43
6	19	116	88			
7	34	21	30	56	18	41
8	50	110	134			
9	53	134	74	73		
10	70	51	30	36		
11	112	50	23	62	35	14
12	113	27	54			
13	138	77				
14	153	38				

Figure 4.5: Parallel cell transfer and study of exosome secretion rate heredity through cell genealogy (continued) (C) A demonstration of cell translocation. Four micro colonies (80 µm diameter each) were translocated to form single-cell arrays at the corresponding positions on the receiving cell culture chip. (D) The exosome secretion rates of daughter cells from 14 parental cells. Cells secreting less than 60 exosomes in 3 hours were labelled as Low EX (blue) and cells secreting more than 80 exosomes in 3 hours were labelled as High EX (red). Those cells that secreted 61 to 80 exosomes in 3 hours were considered to have an intermediate secretion rate (grey). (E) A table to summarize the exosome secretion rate of 14 parental cells and their descendants.

4.6 Relation between exosome secretion rate and gene expression

Using the TransSeA technique, single cells from Low EX and High EX group were randomly picked up for single cell qPCR analysis. Cells were lysed in 11ul lysis buffer (50mM Tris HCl pH 8, 140mM NaCl, 1.5mM $MgCl_2$, 250µl IGEPAL) containing 1mg/ml BSA. RNAsin Ribonuclease Inhibitor (Promega, Madison, WI) was added immediately prior to cell lysis. Cells were lysed by gentle rocking at room temperature for 10 minutes, after lysis cells were maintained on ice.

cDNA synthesis was performed using iScript Advanced cDNA synthesis kit (Bio-Rad, Hercules, CA) per manufacturer's instructions. cDNA was preamplified using Taqman Preamp Master mix (ThermoFisher Scientific, Santa Clara, CA) per manufacturer's instructions. Quantitative PCR was performed to assess the expression of OLIG2, MYC[46], 18S rRNA and GAPDH SsoAdvanced Universal SYBR Green Supermix (Bio-Rad).

Gene expression analysis of OLIG2 and MYC were performed using the 2-ΔΔCt method. For each cell, the number of exosomes, expression level of MYC and OLIG2 were calculated. The 2D graphs were generated using GraphPad Prism Software version 5 (GraphPad, La Jolla, CA) and the 3D data were plotted using Matlab.

As shown in Figure 4 A, the two distinct model distributions roughly divide GBCs into two groups of different EX secretion rate and expressions of MYC and

OLIG, the genes that play a critical role in maintaining and defining glioblastoma cancer stem cell state. Figure 4 B and C show the linear correlation of MYC and OLIG to EX secretion respectively.

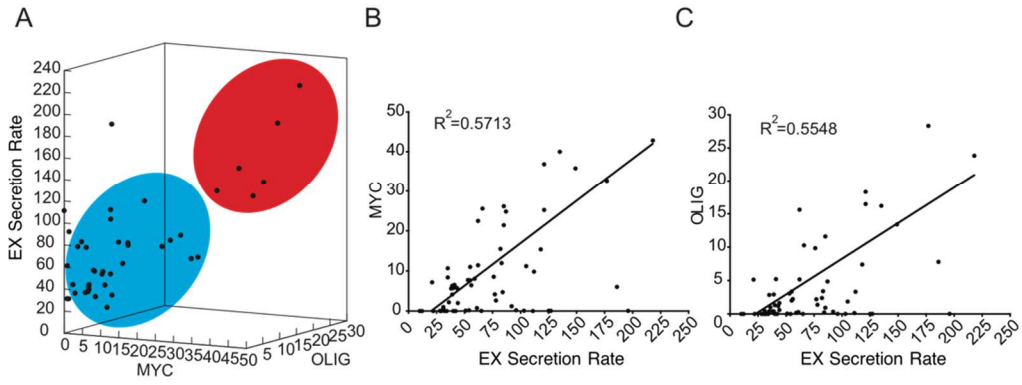


Figure 4.6: Relation between exosome secretion rate and gene expression. (A) EX secretion rate and expressions of MYC and OLIG shows two distinct model distributions of GBM3. (B) Linear correlation of MYC to EX secretion. (C) Linear correlation of OLIG to EX secretion.

4.6 Summary

To summarize, we present a single-cell Translocation Secretion Assay (TransSeA) that offers a myriad of unique capabilities for quantitative investigation of single cell properties. The open platform of TransSeA allows easy access and control of microenvironments, supports cell tracking, time lapse analysis, as well as parallel translocation of single cells and single-cell derived micro colonies based on user-defined criteria and biomarkers. The TransSeA fills the gap between standard FACS-based cell sorting and high throughput sequencing in the single-cell work flow. By way of examples, we use TransSeA to study the relations between cell exosome secretion rate and its life cycle, genealogy, and gene expressions, with the purpose of demonstrating the unique capabilities of TransSeA for quantitative analysis of single cell biology.

This chapter is based on and mostly a reprint of the following papers: Yu-Jui Chiu1, Wei Cai1, Valya Ramakrishnan, Yihuan Tsai, Clark Chen, and Yu-Hwa Lo. An Open Platform for Single-Cell Translocation and Secretion Assay (TransSeA). Nature Biotechnology. Submitted 2017. The dissertation author was the primary investigator and author of this paper.

Chapter 5

Conclusion

A single-cell assay has been developed to be applicable to multiple cell types, friendly for cell loading and operation, supportive for time-lapsed studies, and capable of noninvasive and quantitative analysis of molecular markers and vesicles secreted by single cells. The device is simple, low cost, versatile, and has moderately high throughput to support most single-cell studies. Notably, this platform has demonstrated its ability to support excellent single-cell viability with continuous data up to 96 hours. It has also shown its capability to quantify the various exosome secretion rates of single cells under different in-vitro conditions. In these studies, CD63 positive exosomes secreted by single cells in a 2D array were captured regularly, enabling measurements of exosome secretion rates by individual cells. Two additional surface markers, CD9 and CD81, were used to study the exosomal expression levels for cells under different external perturbations such as drug treatments, pH changes, and growth factors. It is also demonstrated that the capabilities and versatility of the single-cell assay, the results of studies, including the effects of paclitaxel treatment, acidity, and TGF-β on MCF7 and MDA-MB-231 breast cancer cells, have provided valuable biological insight by the quantitative data at single-cell resolution.

By combining parallel cells translocation to the single-cell assay, the single-cell Translocation Secretion Assay (TransSeA) offers a myriad of unique capabilities for quantitative investigation of single cell properties. The open platform of TransSeA allows easy access and control of microenvironments, supports cell tracking, time lapse analysis, as well as parallel translocation of single cells and single-cell derived micro colonies based on user-defined criteria and biomarkers. The TransSeA fills the gap between standard FACS-based cell sorting and high throughput sequencing in the single-cell work flow. By way of examples, TransSeA is used to study the relations between cell exosome secretion rate and its life cycle, genealogy, and gene expressions, with the purpose of demonstrating the unique capabilities of TransSeA for quantitative analysis of single cell biology.

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