Biological Sciences

A Single Cell Assay for Quantifying Extracellular Vesicles from Single Cells

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Heterogeneity of a cell population has been considered a fundamental property of cellular system. Single cell analysis is essential to understand the variation within a heterogenous cell population; however, most existing single cell analysis methods can only provide a glimpse of cell property at specific time point, unable to provide phenotypic information varying in time. Here we review single cell analysis assays we developed to monitor single cell behaviors and cell secretions over time. The assays also provide the capability of translocating cells to another substrate for downstream analysis of selected single cells.

Single Cell, Single-Cell Assay, Extracellular Vesicle, Exosome, Cell Behavior

Introduction

One of the key challenges of biology is to understand how individual cells process information and respond to perturbations. Much of our knowledge is based on ensemble measurements. However, heterogeneity has been widely observed and considered to be a fundamental property of cellular systems[1,2]. Thus, the ensemble behaviors of a population cannot faithfully represent the behaviors of individual cells, especially those "outliers" that can play significant pathological roles such as drug resistance and cancer metastasis. On the other hand, clonal populations of cells exhibit substantial phenotypic variations. Such heterogeneity can be essential for many biological processes and is conjectured to arise from stochasticity, or noise, in gene expression[3].

To address heterogeneity of cell population, many single cell analysis methods have been demonstrated, including, for instances, single cell Raman Microspectroscopy [4–6], a number of acoustic wave techniques [7–10], and a variety of microwell or microfluidic approaches [11–18]. However, these approaches can only investigate cell behaviors at a specific time point as a snap shot of cell properties, missing important time lapsed phenotypic information such as cell secretions over a time period. Such time lapsed information can reveal unique properties of single cells and manifest the heterogeneous properties of cells in terms of cell-cell communications and disease progression, such as intratumor heterogeneity and cancer progression[19,20]. Key constitutes of cell secretions include cytokines and extracellular vesicles (EVs). The latter are nano-sized, membrane bound vesicles such as exosomes and are released by all cell types. Most studies in the EVs are performed with samples containing EVs produced by a large number of cells and cell types, hence the unique behaviors of individual cells are often masked[21–27].

The single cell analysis assays [28–30] described in this review not only address the aforementioned issues but also offer the following salient features: (a) culturing and tracking single cell behaviors as well as single cell secretions (b) allowing studies of single-cell genealogy and hereditary properties of cells produced from single-cell derived colonies, and (c) enabling massively parallel transfer of selected single cells and single-cell-derived colonies from one substrate to another for further downstream analysis.

Single cell culture and quantification of EVs secreted by single cell

We have developed a single cell culture chip (Fig.1) consisting of arrays of microwells or microcavities and a supporting layer at the bottom. Each well in the microarrays has 25 μm to 50 μm diameter for different size of cells, and the center-to-center distance between two wells is 400 μm. The microarrays are made of polydimethylsiloxane (PDMS) by direct lithographic lift-off process rather than conventional soft lithography[28]. The supporting layer at the bottom can be a submicron pore sized permeable membrane or a standard tissue culture plate. To load single cells on the chip, cells suspended in the culture medium are dispensed on the chip by direct pipetting (Fig. 1 A), followed by low-speed centrifugation process that guides the cells into the wells (Fig. 1 B). Those cells outside the wells are flushed away by gently pipetting the culture medium over the surface of the slightly tilted chip. With proper control of the cell concentration in the medium, over 70% of populated wells contain a single cell. To collect EV secretion from single cells, an exosome collection glass slide with immobilized antibodies (e.g. anti CD63 antibody) is placed on top of the cell culture chip (Fig. 1 C). The cover slide has fiducials to help register its location relative to the wells on the single cell culture chip[29] so that the collected exosomes on the glass slide can be easily traced to the individual cells that produce them. To allow oxygen and nutrient perfusion during the exosome collection period, a 100 μm thick spacer is placed between the collection glass slide and the single cell culture chip. We have shown that the 400 μm separation between two wells effectively limits crosstalk due to EV secretion from adjacent cells. EVs secreted from each cell are diffused and captured by antibodies on the glass slide (Fig. 1 D). Those captured EVs are then conjugated with another biotinylated antibody, which is subsequently bonded with streptavidin functionalized quantum dots. As a result, one can use a fluorescent microscope to read the number of captured (CD63 positive) exosomes secreted by each single cell by measuring the number of quantum dots over the corresponding area on the glass slide.

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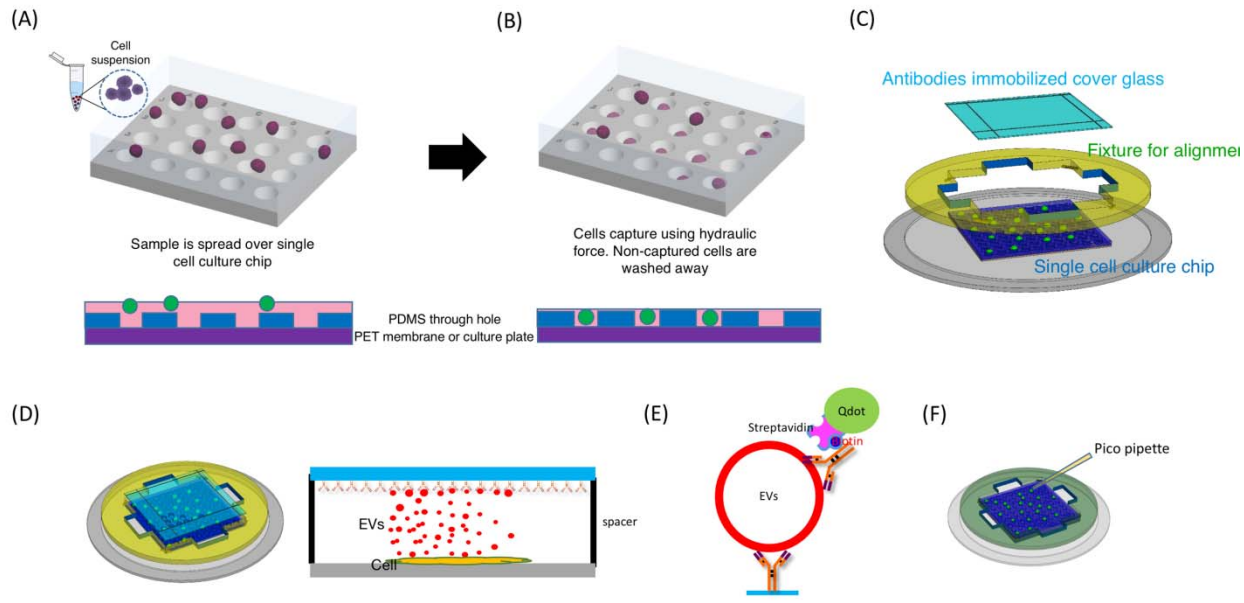

Figure 1. The work flow of single cell culture and quantification of EVs secreted by single cells. (A) Pipetting cell suspension on single cell culture chip. (B) Centrifuge to assist cells to enter microwells. The non-captured cells are flushed away by pipetting. (C) A fixture is used for placement of a antibody-immobilized cover glass on top of the single cell culture chip. (D) EVs secreted by single cells are captured by antibody-immobilized glass slide. (E) Streptavidin functionalized QDs are used to label EV after collection. (F) Selected single cells can be picked up by a pico-liter pipette for downstream molecular analysis such as qPCR and sequencing (schematic not to scale).

Using this assay, we have demonstrated that MCF7 cells and MDA-MB-231 cells have a similar CD63 positive EV secretion rate of 60 - 65 EVs per hour, and MCF10A cells secrete 2.8X more CD63 positive EVs than MCF7. A glioblastoma patient derived cell line, CMK3, shows a much broader distribution of EV secretion rate than the cell lines. Over a 3 hour period, the number of EVs secreted by an individual CMK3 cell varies from less than 10 EVs to nearly 200 EVs[30]. By changing different secondary/biotinylated antibodies, we have used the method to study the expression level of different surface proteins on those CD63 positive EVs. For example, EVs secreted by MDA-MB-231 tend to have higher expression level of CD9 and CD81 than MCF10A[28]. 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 (Fig. 1 F). We have used this technique to selectively pick up single cells according to their exosome secretion profiles and conducted single cell qPCR analysis to find positive correlations between EV secretion rate and expressions of MYC and OLIG2 genes for CMK3 cells[30].

Long term EV collection and cell translocation

Other rarely explored areas for single cell analysis, due to lack of effective tools, are the correlations between EV cargos and cell phenotypic properties and the correlations between single-cell derived progeny cells and their parental cell. Such studies can offer new biological insight to many interesting questions (e.g. whether a cell's EV secretion pattern is stochastic or hereditary from its parent cell). To produce an effective tool to support such research, we have developed a single-cell translocation and secretion assay (TransSeA)[30] capable of collecting EVs secreted by single cells and transferring single cell derived colonies to separated wells of another single cell culture chip, keeping track of the cell locations and their origins.

The work flow of using TransSeA is shown in Fig. 2. To analyze EV cargos from single cells, we need to collect single cell secretions over a long period (typically over 24 hours) to obtain a sufficient number of EVs (>10,000). To support EV collection over such long period without affecting cell properties, the single cell culture chip is modified from the previous design by adhering the single cell culture chip to a permeable membrane (e.g. 0.8um pore size) (Fig. 2 A). While antibody-specific EV secretion rate is characterized by the cover glass on the top as discussed previously, EVs at the bottom surface are collected in the well through a thin (100um) porous membrane. Using this technique, we have demonstrated that microRNAs carried by EVs can be quantified using droplet digital PCR (QX200™ droplet digital™ PCR system, Bio-Rad). The preliminary results showed positive correlations between miR-21 copy numbers carried by EVs and the secretion rate of EVs[30].

Cell secretion being an important phenotype of cell-cell communication, studying the cell secretion change across parental and daughter cells could help understand the translational change of cell heterogeneity. The TransSeA assay can transfer single cells or single cell derived colonies from the original chip to a new single cell culture chip in two steps. At first, we place two chips facing each other separated by a spacer in a fixture with a fluid inlet and outlet. By a hydraulic pressure driven flow, the cells in one chip are pushed out of their original wells and transferred to wells in another chip. The cell motions follow the streamline controlled by the negative pressure at the bottom. With different single cell culture chip designs, we can either translocate single cells to single wells, or disperse each cell within a single-cell derived colony to individual wells (Fig. 2C). After single cell qPCR analysis, we have discovered the stochastic pattern of EV

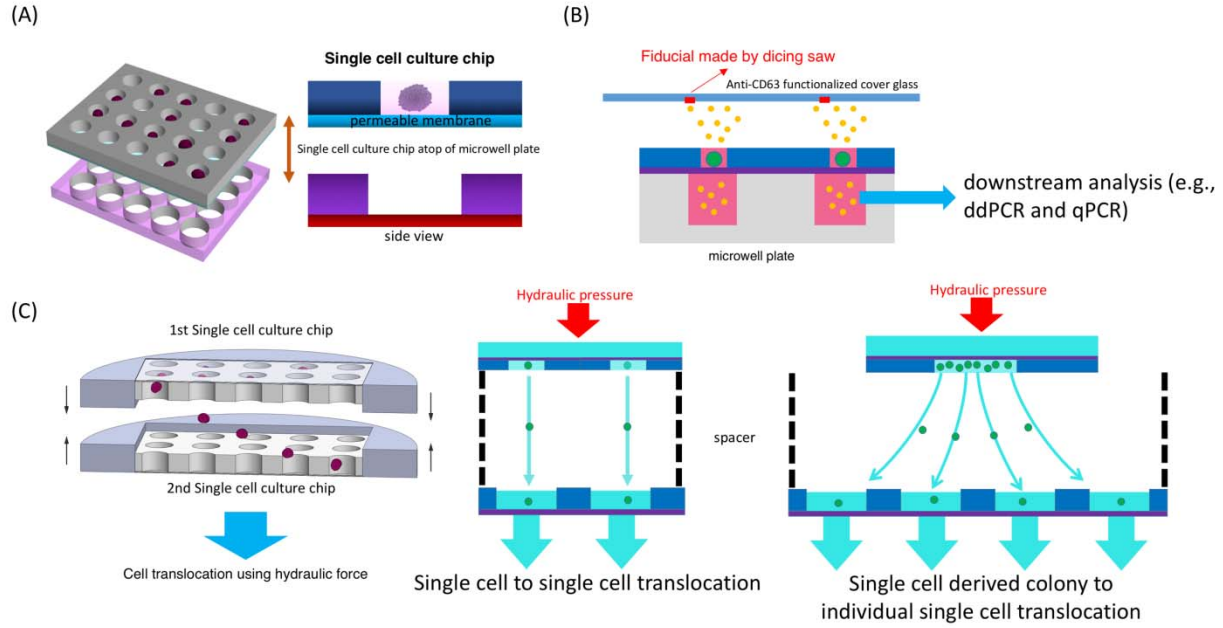

Figure 2. The work flow for EV collection over a long (>24 hours) period and parallel cell translocation. (A) Two single cell culture chips of the same well-well separation for cell translocation. (B) Cell secretion can be short-term (1-3 hours) characterized by a functionalized cover glass on top and long-term (>24 hours) collected by the well at the bottom. (C) Single cell or single cell derived colony can be translocated to a new single cell culture chip by applying hydraulic force (schematic not to scale).

secretion rate between two generations of CMK3 cell lines. We have found that a low EV rate appears to be the normal state for CMK3 cells since EV secretion rate tends to be low even for progeny cells produced by parent cells of a high EV secretion rate. On the other hand, occasionally some cells produced by low EV secretion parent cells possess a high EV secretion rate. There is no clear pattern to show that EV secretion behaviors are hereditary.

Summary

We have reviewed single cell assays for time-lapse characterization of secretions from single cells, using single-cell EV secretion studies for proof of concept. Then we present a more advanced design, single-cell translocation and secretion assay (TransSeA), with expanded capabilities. In addition to all the functions of the first design, the TransSeA assay allows continuous collection of EVs from single cells for studies of EV cargos and parallel translocation of single cells and single-cell-derived colonies to a new single cell chip to support studies of progeny cells, which is a unique capability to enable investigations of phenotype information across cell generations with single cell resolution.

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