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Single-cell Isolation Devices: Understanding the Behaviour of Cells

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Abstract

Practical usefulness in biological and clinical settings has become an important focus during the development and implementation of new instrumentation and assays. These developments have allowed it to become possible to determine gene- and protein-content, as well as mutations within the transcriptome of a single cell. In order to be able to reach the full potential of the available instrumentation and assays, it is required to develop a method to first isolate an individual cell. This review serves as an overview of available techniques for single-cell isolation by describing the biological information about a single cell that can be obtained from each technique.

Introduction

The invention of the microscope in 1676 by Anton van Leeuwenhoek introduced the concept of studying how the human body is constructed. From here, Robert Hooke coined the term "cell" as the basic building block for all living species. The discovery of the cell was instrumental to further our knowledge about many aspects of the human body as focus was then shifted from whole tissue to cell suspensions so that analysis could be undertaken on cells with prior knowledge of their origin. The development of the first immortal cell line in 1951, the HeLa cell line, showed that it was possible to investigate cells with respect to time to better understand how the cells respond to a particular treatment. Using this understanding of how to encourage the continuous culture of cells, a wide range of cell cultures originating from various parts of the human body were created to learn how different parts of the human body react to external influences (Norris and Ribbons, 2006). From these cell lines, it has been noticed that mammalian cells from the same cell line can respond differently to the same procedures to analyse them (Andersson Svahn and van den Berg, 2007). This means that information collected from cell populations represents averaged values and can potentially mask rare but important events (Di Carlo and Lee, 2006). This has led to a shift from studying cells within cell lines down to individual cells in order to learn how each cell behaves and communicates with its neighbours. A cell can for example be monitored as it migrates or divides into two cells. The understanding of cell migration would give insights into the nature of tissue repair after injury whilst cell division is of interest in for example, cancer, due to the uncontrolled

rate of cell proliferation of a cancer cell compared to a normal cell. Furthermore, the dynamic study of living cells can increase the understanding of the interconnecting molecular events continually taking place in each cell as it responds to external influences such as a particular treatment or other cells. As the human body contains a variety of cells such as stem cells, blood cells and tissue cells, that all vary in their behaviour, a wide range of single cell devices have been developed that enable behavioural information for all types of cells to be gained.

Methods for Single-Cell Isolation

Serial Dilution

Just like with any problem facing concentrations or amounts that are too high, the first solution that tends to come to mind is to dilute the sample. This was no different with cells, with the first methods to reach lower numbers of them being achieved by successively diluting cell solutions until it was possible to microscopically observe the occasional aliquot that contained one cell. However, tracking these single cells in bulk amounts of volumes required to perform serial dilution can be difficult and therefore analysing these cells may not be possible. For this reason, a method to trap these cells is required so that continuous analysis of them is possible.

Microwell Trapping

Once a cell-rich sample had been diluted such that an aliquot containing a single cell was made available, a means to be able to investigate the behaviour of that cell is required. In other words, a method to trap the individual cells is required. The most common method to trap cells is by placing them in an array of wells. Since most of these cell lines rely on the cells adhering to a surface in order to proliferate, they can be easily identified once they have adhered to the bottom of the well. The physical wall placed around each sample to protect it from cross-contamination with other samples allows for multiple parallel studies to be undertaken within each adjacent well, increasing the amount of information that can be obtained. As the number of cells to be studied decreases, so do the associated volumes. To facilitate the smaller volumes used, wells of continuously decreasing size are being developed. At present, the most commonly used is a 96-well plate. With these wells having a surface area of 0.32 cm^2 (about 100 000 times the area of an adhered cell), they are more suited to the study of small colonies (hundreds of cells) rather than an individual cell.

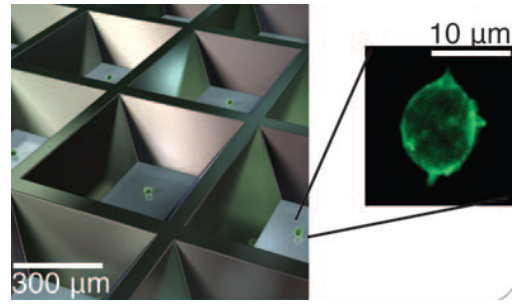

Figure 1: A representation of microwells containing single cells and (inset) a microscope image of an adhered human stem cell. Reproduced from Lindstrom et al. (2009).

The logical progression is to make wells that are similar in size to that of the spread cell (Figure 1). Despite its miniaturisation, this approach is still quite simple and is therefore quite popular with overviews of the varying methods being reviewed in (Walling and Shepard 2011, Lindstrom and Andersson-Svahn 2011). The smaller sizes

and volumes within these microwells means that shorter diffusion distances are present and therefore the immediate effect of an external influence on a particular cell can be examined. Furthermore, these wells have been created with varying characteristics such as well shape, rounded (Wood et al. 2010, Rettig and Folch 2005, Tokimitsu et al. 2007, Ostuni et al. 2001), hexagonal (Taylor and Walt 2000, Deutsch et al. 2006) and square (Chin et al. 2004, Revzin et al. 2005, Lindstrom et al. 2009), number of wells (100's: Taylor and Walt 2000, Ostuni et al. 2001, Lindstrom et al. 2009; 10,000's: Chin et al. 2004, Revzin et al. 2005, Rettig and Folch 2005, Deutsch et al. 2006; or 100, 000's: Tokimitsu et al. 2007) and fabrication material (glass: Deutsch et al. 2006, Lindstrom et al. 2009; silicon: Tokimitsu et al. 2007; polydimethylsiloxane (PDMS): Rettig and Folch 2005, Ostuni et al. 2001; and polyethylene glycol (PEG): Revzin et al. 2005).

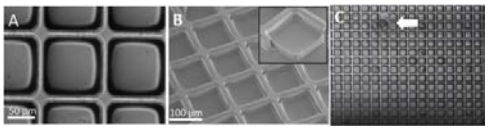

Figure 2: (A) Brightfield and (B) SEM images of microrafts. Inset shows a side view of a raft with PDMS partially removed. (C) Removal of a predetermined microraft after cell seeding. Reproduced from Gach et al. (2011) with permission from AIP Publishing LLC.

Part of the driver for these different variables is finding conditions for the optimisation of cell viability over moderately longer time periods, enabling the viewer to gain information with regards to the affects of a particular external influence over a period of 8 days. Despite it being desirable for aiding the initial colonisation of a single cell, the small surface area of the microwells does not facilitate the growth of these colonies and cell viability decreases as overcrowding in each well occurs. Since many influences can cause the cells to display symptoms only months, or even years after they are affected, the ability to analyse these cells over much longer time periods is required.

Cell Microsystems (North Carolina) had this in mind when they created their IsoRaft system, which is a microwell plate made of a compliant polymer substrate. In the bottom of each well is a concave-shaped tile-like object made of hard polymer-material (polystyrene or epoxy resin) that has been called a microraft (Figure 2A and B). This concave-like shape of the microraft causes the cells to adhere at the bottom when they are trapped in the microwells. Much like the other microwell methods, a single cell can be selected and analysed to determine the immediate effects of an external influence on the behaviour of the cell. For long-term effects, a selected cell can be monitored until it forms a small colony. Once the microraft begins to get crowded with cells, a needle can then be inserted into the compliant polymer substrate adjacent to the microraft containing that colony, moved around it and in doing so, removing the microraft containing the colony of interest from the microwell (Figure 2C) (Wang et al. 2010). However, an issue faced when using this IsoRaft system was the difficulties associated with trying to recover this released microraft. Cell Microsystems overcame this issue by incorporating magnetic nanoparticles[1] into the microrafts and then collecting them magnetically

[1] Magnetic nanoparticles are particles with a diameter of less than 100 nm and contain a magnetic iron oxide core. This magnetic core allows them to be recovered using a magnet.

(Gach et al. 2011). These collected microrafts (containing the cells of interest) can then be placed into a flask with a larger surface area than that of the microwells. This larger surface area allows for more replication cycles and therefore more long-term (up to years) effects, such as a better understanding of the mutation rates of cells. However, these methods typically focus on the behavioural analysis of these cells. This is because limited techniques, with the exception of cytoplasmic staining, can be used to gain structural information about cells that are trapped on a surface.

Microwells have shown to be a simple and effective method to isolate an individual cell. The next step is to be able to provide a technique that can acquire the individual cells in an unbound state such that the newly-developed assays and instruments can be used to gain further information about the behaviour and contents of single cells as they are exposed to an external influence.

Droplet Trapping

One way to be able to have tracked single cells in an unbound state is by enclosing them in droplets of low volumes (fL to nL), forming micro-chambers for individual reactions. High-frequency (Hz-kHz) droplet generators in microfluidic devices[2] form monodisperse drops of water in an inert and immiscible carrier fluid (oil). Controlling the number of loaded cells per drop has been a barrier for droplet-based single-cell analysis, due to the stochastic limitations of single-cell loading resulting in *ca.* 30% of single-cell occupancy (similar to many microwell approaches). A demonstrated way to overcome this limitation has been to evenly space cells in a microchannel to make sure that the cells entered the drop generator with the same frequency as drop formation (Figure 3; Edd et al. 2008). As with limited dilution in general arraying techniques, empty droplets are often preferred rather than overloading droplets with several cells. The droplets can thereafter be merged with other droplets, (Chabert et al. 2005) split into two (Link et al. 2004) or dielectrophoetic (DEP) sorted (Ahn et al. 2006).

Figure 3: A micrograph depicting the encapsulation of single cells within nanodroplets. The ordering of cells entering the droplet chamber increases the likelihood of a droplet containing a single cell. Scale bar = 150μm. Reproduced from Joensson and Svahn (2012) with permission from Wiley and sons.

There are two strong advantages of this technique: 1) since each cell is kept within its own separate droplet, isolated from other droplets, the risk of cross-contamination decreases and 2) the even lower volume of liquid surrounding each encapsulated cell when compared to microwells, results in even more accurate short-term information. Weitz and co-workers showed an example of such an application by incubating single hybridoma in 33 pL drops of media, giving rise to secreted detectable concentrations of antibodies after 6h (Koester et al. 2008). Another example of an application of cell encapsulation demonstrates laser-induced cell lysis within droplets followed by monitoring the activity of β-galactosidase enzyme from a single cell (He et al. 2005). Samuels and co-workers (Brouzes et al.

[2] A microfluidic device involves fabricating chambers with micrometre dimensions that are designed to accurately control the flow of volumes of liquids in the millilitre range.

2009) have extended on these applications with an integrated droplet-based workflow for conducting a mammalian cell cytotoxicity screen at high throughput. Cells were kept viable for four days (though cell proliferation was only detected during the first 24h) and a drug library was screened for their cytotoxic effects against cells from a myeloid cell line. Most importantly, the unbound nature of these single cells within a droplet allows structural information to be gained from them. Enzyme amplification was used to detect low abundance cell-surface biomarkers, CD19 and CCR5 on single U937[3] cells (Joensson et al. 2009) and shows the potential of this technique to be used to decipher the expression of genes, and even potentially, mutations within these genes as the cells become affected. In order to get more accurate information about cells with respect to time, issues such as 1) changes in the droplets such as coalescence, nutrient depletion or the accumulation of toxic metabolites are obstacles that need to be considered before robust analyses over longer periods of time can be achieved. Despite minor success with regards to this (Clausell-Tormos et al. 2008) these issues still hinder most methods that have adopted this technique and 2) the fact that each cell is isolated as many influences affect the way that a cell responds to its environment and communicates with its neighbouring cells. Merging droplets can gain information with regards to this but with limited control over which droplets to merge, this information can only be elementary. In essence, droplet trapping has served as a powerful means for being able to gain short term information about the behaviour or contents of a single cell. The next step is to be able to get information that more accurately mimics the conditions within the human body. Namely, the behaviour of a single cell within a network of cells over extended periods of time.

[3] U937 cells are a commonly used cell line used in biomedical research. They were isolated from the histiocytic lymphoma of a 37-year-old male patient.

Hydrodynamic Trapping

These requirements are addressed by hydrodynamic trapping. This technique involves flowing a cell solution through a microchannel that contains microstructures that trap individual or clusters of cells. The flow of medium through the channel after the cells have been trapped means that the nutrients for the cells are being replenished, allowing for the cells to be kept in a viable state for longer periods of time. Furthermore, hydrodynamic trapping has shown a high selectivity when it comes to pairing individual cells together to see how cells interacts with each other when placed in a range of environments. It comes on the back of initial work carried out by Lee and co-workers (Di Carlo et al. 2006b), who first showed that it was possible to generate single-cell arrays using U-shaped hydrodynamic trapping structures with geometries that are biased to trap only single cells (Figure 4A). Lee and co-workers (Di Carlo et al. 2006a) used these U-shaped arrays to report novel data on the single-cell concentration distribution of carboxylesterases within three different human cell lines, as well as on the inhibition of intracellular esterases by the non-specific inhibitor nordihydroguaiaretic acid. Benavente-Babace and co-workers (Benavente-Babace et al. 2014) further showed that it was possible to treat a subpopulation of the single cells captured with these U-shaped geometries. From these initial studies, hydrodynamic traps have been used to capture pairs of single

cells (Figure 4B) to gain a further understanding of cell-cell interactions such as cell fusion (Skelley et al. 2009) and cellular uptake of secreted proteins from neighbouring cells (Chen et al. 2014). This information can be coupled to single-cell studies and can give valuable insights into how a disease requires the presence of regular cells in order to be active.

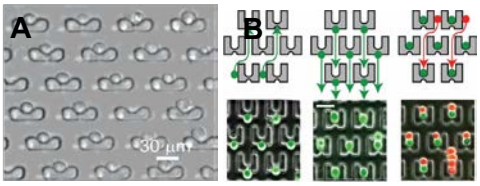

Figure 4: (A) Arrayed single-cell culture within U-shaped sieves. Reproduced from Di Carlo et al. (2006a) with permission from the American Chemical Society.
(B) Paired single 3T3[4] cells within a U-shaped sieve. Green-stained cells are first loaded 'up' towards the smaller back side capture cup (left panel). The direction of the flow is then reversed and the cells are transferred 'down' into the larger front-side capture cup two rows below (middle panel). The red-stained cells are then loaded in from the top and cells are captured above the first cell type (right panel). Scale bar is 30µm. Reproduced from Skelley et al. (2009) with permission from Nature Publishing Group.

The hydrodynamically-based trapping methods discussed thus far show that it is possible to study the interactions between two individual cells of a different type. Zhang et al. (2014) have improved on the modularity of this method by creating a hand-held single-cell pipette. The pipette contains positive pressure and negative pressure channels, along with a tip that contains associated channels. Single-cell transfer is achieved through four steps: preparation, capture, washing and release (Figure 5D). Initially, the channels and tip are filled with cell-free medium. Cells are then sucked up into the tip where a single cell is captured by a hook located within it (Figure 5A) whilst the pipette is quickly transferred to cell-free medium to wash the remaining cells into the negative pressure channel. The captured single cells are easily released into nanoliter droplets by applying a gentle pushing force to the positive pressure channel. Subsequently, single-cell droplets are conveniently transferred into designated containers, such as standard 96-/384-well plates, Petri dishes, and vials.

The versatility of this hand-held single-cell pipette means that any single cell can be isolated and then its genetic and cytoplasmic contents can be determined or multiple single cells can be placed next to each other to gain knowledge about how a network of cells communicate.

These single-cell methods showed that it was possible to trap single or paired cells. To build from this, the ability to get behavioural and structural information from these isolated individual cells lies within the development of newly-developed instruments and assays.

[4] 3T3 cells are a fibroblast cell line derived from Swiss albino mouse embryo tissue.

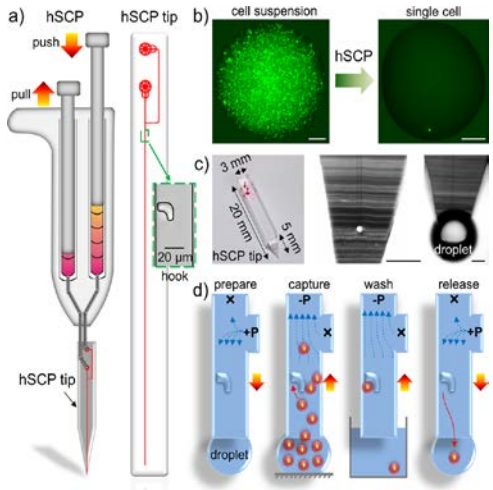

Figure 5: Design and mechanism of the hand-held single-cell pipette (hSCP).
(a) The hSCP involves one dual-channel pipette and one hSCP tip. A magnified hook for single-cell capture is shown.
(b) A single calcein-labeled SK-BR-3 cell is isolated directly from a dense cell suspension by hSCP.
(c) The hSCP tip with a conical end and two magnified tip ends shown before and after extrusion of aqueous solution.
(d) Work flow for single-cell isolation using hSCP. Scale bar in (b) and (c) = 20µm. Reproduced from Zhang et al. (2014) with permission from the American Chemical Society.

Single-cell Analysis Instruments and Assays

Due to the important nature of the information that can be gained, several single cell analysis methods have become available. Most of the available protocols are focused on either the nucleic acid content (PCR-based methods) or on cytoplasmic protein level (cytometric-based methods).

Polymerase Chain Reaction (PCR)-based Analysis

PCR is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. This amplification process has allowed it to become a powerful technique for the genetic screening for the small numbers of cells, towards the single cells isolated from single-cell isolation devices.

To be able to get a further understanding of the structural make-up of individual cells as they are exposed to an external influence, it is required to not only increase the number of copies of a particular gene, but also to increase the number of genes to be amplified. For this reason, gene analyses has leaped forward in the mid 1990s with the development of new amplification methods such as single-primer isothermal amplification (SPIA) Ma et al. (2013) and the rise of high-throughput RNA/DNA sequencing, or RNA/DNA-seq, which gives the sequences of thousands of cellular RNAs/DNAs from a single cell at once, giving rise to a new field of single cell sequencing. Due to its high impact, the field has seen a proliferation of methods for performing single-cell RNA/DNA-seq (Hashimshony et al. 2012, Tang et al. 2009, Ramskold et al. 2012, Islam et al. 2011, Sasagawa et al. 2013) and have been reviewed in detail (Sandberg 2014, Tang et al. 2011). More detailed information about the behaviour of cancerous cells has already been obtained, such as the re-evaluation of their mutation rates. Bulk-sequencing studies have estimated that the mutation rate across many human cancers is, on average 210-fold higher than normal cells (Bielas et al. 2006, Bielas and Loeb 2005). However, single cell sequencing has shown

that an endoplasmic reticulum-positive breast cancer cell did not have an increased mutation rate relative to that of normal cells, whereas a triple negative (endoplasmic reticulum, pathogenesis-related and human epidermal growth factor receptor 2) breast cancer cell showed an approximately 10X increase (eight mutations per cell division) relative to that of normal cells (Wang et al. 2014). Furthermore, differences within the transcriptional profiles of breast cancer samples taken from patients have been recorded (Powell et al. 2012), point mutations within targeted genes identified (Heitzer et al. 2013) and whole genome sequencing of single cancerous cells to trace how the tumour evolves (Navin et al. 2011).

Despite the important information that PCR-based methods have already supplied, the amount concentration of a gene is only in the order of 10^{-12} M (~10 pg per cell) while the total protein content is as high as 10^9 molecules per cell (hundreds of pg). It has been estimated that a cell contains more than 100,000 different proteins, ranging from <200 copies of many receptors, 1,000–10,000 copies of signalling enzymes, to 10^8 copies of some structural proteins (Cooper and Hausman 2007). For this reason, limiting the investigation to just the genes that are present in the nucleus results in only a partial understanding of the structural make-up of individual cells.

Cytometric-based Analysis

Cytometry involves measuring the characteristics of cells, focusing on the enumeration and understanding of specific proteins on the cell surface or in the cytoplasm. For this reason, cytometric-based analytical methods are considered a suitable avenue to broaden an investigation of single cells beyond their nucleic material.

Image cytometers involve the use of microscopes to acquire highly-resolved images of the single cells isolated from the previously-mentioned devices and have the potential to gain a high level of information about the structural make-up and behavioural patterns of a single cell or network of cells as an external influence is applied to them. Initially, identifying rare cells by microscopy was highly laborious with the accuracy and sensitivity being a subject of the fatigue encountered by the viewer. However, throughput and accuracy were improved as the newly-introduced digital camera began to rise in popularity in the late 80s/early 90s (Mansi et al. 1988, Lee et al. 1989, Mesker et al. 1994) and were incorporated into the first digital image microscopy systems. Since then, newer systems have been developed with the aim being to increase the resolution and speed and therefore acquire more detailed information about the cell (Kraeft et al. 2004, Krivacic et al. 2004, Hsieh et al. 2006). However, the biggest advancement of this technique came with the incorporation of sensors that monitor the *xy* position of the slide on the computer-controlled motorised microscope stage, which moves at 0.5 μm-steps per each laser scan, perpendicular to the scan (Pozarowski et al. 2006). This enables the detected cells of interest (either by scattered laser light or specimen-emitted fluorescence) to be relocalised in sequential measurements. This ability to get temporal information has been used to 1) discriminate, through cell morphology, between the genuine apoptotic cells and 'false-positive' cells in peripheral blood and bone marrow of leukemic patients undergoing chemotherapy (Bedner et al. 1999), 2) reveal translocation of proteins throughout the cell during mitosis (Kakino et al. 1996), 3) measure kinetic reactions within individual cells in large populations

(Bedner et al. 1998, 4) enumerate cells at the completion of therapy to determine the likelihood of early relapse (Pachmann et al. 2008).

Single-cell analysis techniques have already shown promise to reveal important information about the behaviour of cells within the human body and will continue to reveal further insights as spatial and temporal resolution is improved with later generations of analytical assays and instruments.

Conclusions and Future Perspectives

A variety of methods for single-cell isolation have already been developed, and by combining these isolation techniques with newly-developed single-cell assays, a focus has been directed towards solving clinical problems. The key to gaining more valuable clinical information of this nature lies within the development of single-cell isolation strategies with a higher degree of control over which single cell to analyse. The shift in focus down to one individual cell has led towards a better ability to understand cellular heterogeneity. To gain a better understanding of important events within the human body will require the cells that are responsible for these events to be determined prior to their analysis. For this reason, the next generation of single-cell isolation strategies are required to incorporate a selective element to discriminate between different types of individual cells.

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