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Table of Contents Entry

Frontier microfluidic techniques for short and long-term single cell analysis

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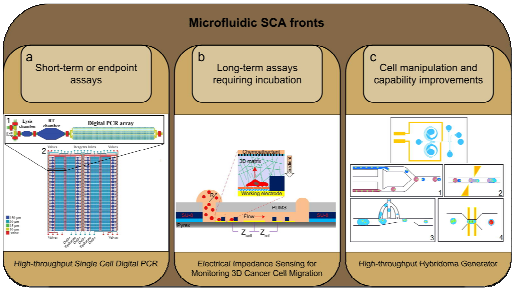
This review details the frontier microfluidic platforms for single cell analysis, highlighting technological improvements and cell analysis capabilities.



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Frontier microfluidic techniques for short and long-term single cell analysis

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Here we review the frontier microfluidic techniques for single cell analysis (SCA), important for research of many biological systems. Microfluidics provides high-throughput, high-resolution experiments at low cost and reagent use, making it especially useful for single cell analysis. Recent advancements in the field have made SCA more feasible, improving device throughput and resolution, adding capabilities, and combining different functions to bring forth new assays. Developments in incubation have allowed for long-term cell tracking assays to be performed with single cell resolution. The ability of systems to provide chemical isolation or prolonged growth of adherent cells is also discussed.

Introduction

Heterogeneity between individual cells in response to external stimuli exists even between genetically identical populations, however this information is generally lost in common bulk cell assays, relying on ensemble averages to understand cellular phenomena. Single cell analysis (SCA) is an important and emerging field that gives insights into heterogeneity between cells and advanced cellular processes at high resolution, important for cancer research, regenerative medicine, immune system research and diagnostics, as well as for the production of therapeutics[1-6].

Microfluidics has proven to be a leading tool for single cell analysis since device dimensions are on the same scale as those of cells, allowing for precise fluid and cell manipulation at high throughput. In addition, microfluidic assays typically require little reagent consumption, and if cells are chemically compartmentalized, cellular microenvironments can be controlled with high spatial and temporal resolution. Moreover, microfluidic SCA systems require low cell numbers, making them ideal for studying rare species of cells.

Previous reviews have discussed cell analysis[1–3] and culture[4] capabilities, assessed the capability to replace larger scale techniques[5,6], and assessed the capability to aid in the production of therapeutics[7]. This review details the frontier microfluidic platforms from the last two years for single cell analysis (SCA) with respect to the technological improvements and cell manipulation capabilities that they provide along with the assays that they support. We specifically emphasize the difference between single cell analysis platforms that do or do not compartmentalize single cells as well as support adherent cells or not, and explain how these two features contribute to the efficacy of both short and long term analysis schemes.

Recent microfluidic cell manipulation and capability improvements were made in cell displacement, droplet and cell sorting, electric field cell manipulation, micropipettes, device optimization and software for droplet image analysis. Frontier developments in short-term analysis systems discussed here analyze cell DNA, RNA, intracellular proteins and molecules, heterogeneity, deformability, and cytotoxicity. Developments with long-term analysis systems involve measuring cell secretion, formation of stationary culture arrays, reducing medium evaporation, improved cell-cell contact and interaction studies, as well as prolonged tracking of growth and other parameters under various conditions. Adherent cell compatible platforms are scarce but have shown recent contributions with 2D substrates, 3D gels, and mobile micro-substrates.

Chemical compartmentalization of individual cells enables independent high throughput experiments as well as cell secretion assays. Although there are several microfluidic platforms that can support non-adherent cells for long time periods in isolated compartments[7], the majority of multicellular animal cell types require a biocompatible adhesion surface or matrix to mimic their growth environment *in vivo*. In spite of its relevance, we find a lack of methods and improvements thereof for the study of single compartmentalized adherent cells.

Cell manipulation and capability improvements

Microfluidic channel dimensions are on the same scale of those of cells, enabling fine cell displacement[1], achieved using optical, magnetic and electric tweezers, or simply by analyzing flowing droplets and changing the direction of the flow in real time. Optoelectronics, which exerts a lower optical density as compared to optical tweezers, was integrated with a microfluidic device for selective single cell capturing and retrieval based on cells' fluorescent properties[8]. Microfluidic droplet FACS devices can also be used for this purpose. Droplet FACS can sort droplets not only by a fluorescent signal inside the cell but can also separate cells by fluorescent assays[9,10] or selectively collect droplets which contain exactly one fluorescent cell[11], thus surpassing the inherent 37% limit of Poisson distribution. Selection can be done based on secreted antibodies or fluorescent assays. Droplet sorting can also be conducted optically in high throughput on non-fluorescent samples using image-based droplet sorting (IDS)[12].

Other, non-optical single cell techniques are used as well, such as immunomagnetic cell sorting and encapsulation[13]. A less

invasive method for cell displacement is surface acoustic waves (SAW) which exerts lower power density than optical and dielectrophoretic tweezers. It was shown to be able to move cells and even live multicellular organisms in arbitrary 2D paths[14], although versatile cell displacement was not shown inside a complex microfluidic channel which may limit its practicality. Deterministic movements were shown in microfluidic channels for high-throughput multichannel droplet sorting[15], microparticle size separation[16] and bacteria separation from peripheral blood mononuclear cells (PBMC)[17]. Localized cell concentration was also demonstrated using SAW by attracting cells to a point of choice while controlling the attraction radius[18].

Kemna et al constructed a simple microfluidic device which encapsulates single cells, in high yield (77%), with high throughput (2700 cells/sec) using inertial ordering which does not require exterior sorting tools[19]. They embedded this, and additional microfluidic tools to make a platform which generates hybridoma cells with high probability[20] (Fig. 1a). Single cell droplets of two cell types are formed using the early device, mixed to create droplets which have one of each cell type, shrinked to make the cell-cell contact, and then electrofused to form the hybridoma.

Microfluidic electroporation[21] can be formed inside portable droplets by flowing them over two electrodes separated by a distance smaller than the droplet diameter. Creating a field inside a droplet requires low potential, and solves the heating problem due to the large surface to volume ratio. This tool was used for cell transformation with higher efficiency compared to bulk transformation[22] and impedance cell sensing[23]. Fine tuning of the electric field can also be used to chemically isolate the content of the cytoplasm of a single cell[24].

Often the low signal of high throughput devices limits image acquisition. Increasing fluorescent signal is typically achieved by more sensitive microscopy systems or manipulating the biological sample, however this can also be achieved by optimizing the device for increased signal and signal to noise ratio (SNR). Lim et al fabricated a droplet based microfluidic device coupled to a microlens array[25]. By using this approach, and parallelizing over 100 channels, they reached an analytical throughput of 10^5 droplets per second. Galla et al embedded carbon black in PDMS to increase the SNR of UV signal in the analysis area of a PDMS device[26].

Analysis of fluorescent signals from cells in bulk is difficult and typically limited in throughput due to the sample preparation yielding random cell locations thereby forming clumps or overlapping, which can be solved by immobilizing cells in an array. Although droplet microfluidics has many advantages for cell arraying, many assays can be done without droplets in non-compartmentalized cell array platforms, which enable easy, high throughput image processing. Cell immobilization can be achieved by stretchable microwell arrays smaller than the cell size[27], surface treatment[28], DNA probe adhesion[29], electrostatic interaction[30] or

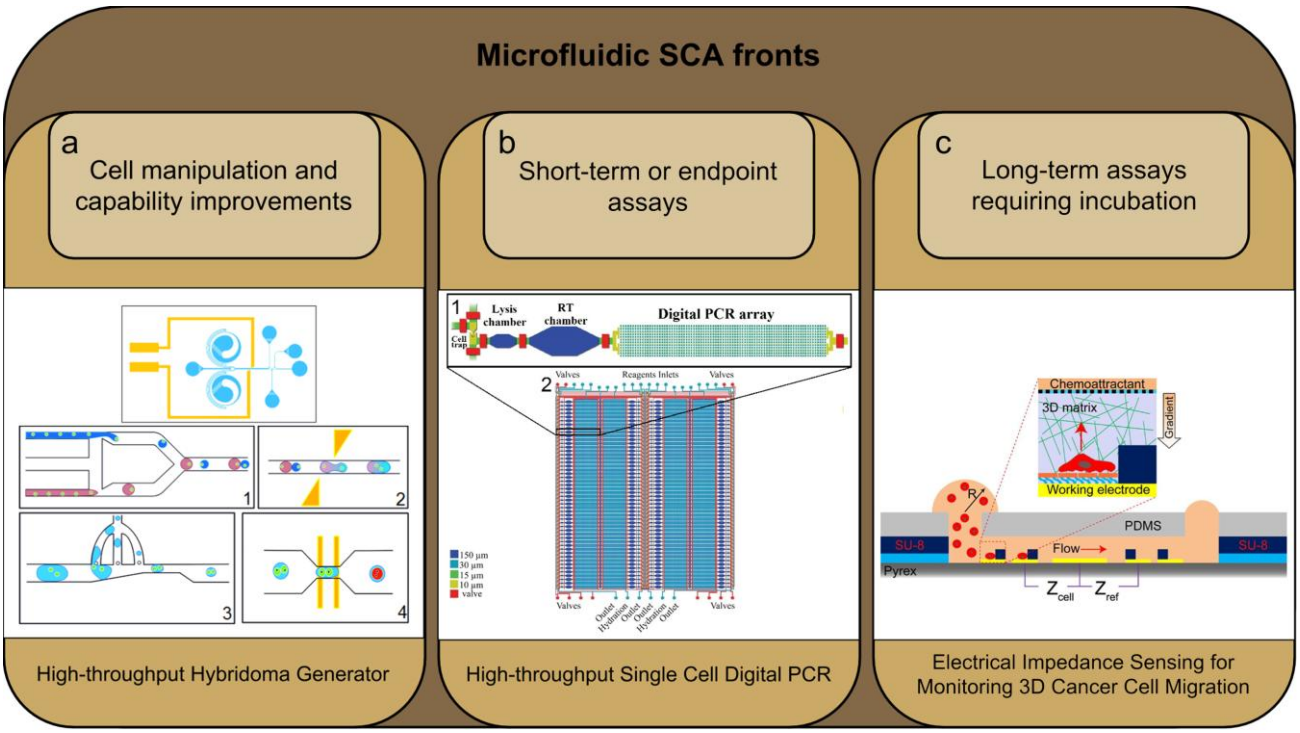

Figure 1 | *Microfluidic SCA fronts.* ***(a)*** *Cell manipulation and capability improvements. Example: Hybridoma generator with an ensemble of four microfluidic tools. 1. Deterministic single cell capturing with high yield (77%). 2. Droplet electrocoalescence. 3. Droplet shrinkage 4. Cell electrofusion.* ***(b)*** *Short-term or endpoint assays. Example: High-Throughput single cell resolution Digital PCR. (1) Schematic for each cell section with lysis and RT chambers in combination with dPCR chambers. (2) The entire device. This device is capable of analyzing 200 single cells in parallel with 1020 PCR reactions for each, making a total of 204 000 reactions.* ***(c)*** *Long-term assays requiring incubation. Example: Tracking cancer cell migration in 3D gel using impedance sensing from microelectrodes.* ***(a)*** *Adapted from Schoeman et al[66], Electrophoresis, 2013[†].* ***(b)*** *Reprinted (adapted) with permission from (A. K. White, K. A. Heyries, C. Doolin, M. Vaninsberghe, and C. L. Hansen, Anal. Chem., 2013, 85, 7182–7190). Copyright (2013) American Chemical Society.* ***(c)*** *Reprinted (adapted) with permission from (Tien Anh Nguyen, Tsung-I Yin, Diego Reyes, and Gerald A. Urban, Anal. Chem., 2013, 85, 11068-11076). Copyright (2013) American Chemical Society.*

open microwells[31,32].

An essential part of high throughput microfluidic data is efficient image analysis. Image analysis can be done in real time and can be coupled to the platform itself for cell sorting[12]. Efforts have also been made for thorough video analysis of droplet morphometry and velocimetry[33]. This program detects and analyzes parameters over time such as velocity, deformation, trajectory, area, fluorescence intensity and can be used as an efficient tool for data analysis, relevant for common microfluidic experiments.

Microchannels were recently used as micropipettes for the delivery of tunable picoliter volumes for single cell chemical stimulation in bulk as well as for single adherent cell and single bacteria retrieval[34–36].

Inlet holes in microfluidic devices are usually created orthogonal to the flow of the system, which results in particle loss due to sedimentation in the inlet area. Lee et al suggest to drill the inlets lateral to channels to prevent variation in particle and cell concentration[37].

While making a point-of-care (POC) tool, one might choose to use paper microfluidics. Using a smartphone as the analysis instrument, Park et al developed a method to measure Salmonella concentration as low as 100 CFU/mL[38].

Short term or endpoint assays

The ability to generate and process small liquid volumes with high throughput including the ability to precisely control them with high spatial and temporal resolution, makes microfluidic platforms relevant for short term or endpoint assays. Since these assays can involve large multistep protocols, multifunctional lab-on-a-chip devices are particularly useful. Additionally, many of these assays involve cell lysis or utilize fast detection or rapid manipulation steps making cell exposure to the device relatively short. Thus, cell biocompatibility and support for adherent cell attachment is less crucial than in long-term assay systems. Lastly, protocols that do not involve cell lysis can support cell retrieval and be coupled with additional microfluidic devices for multiplexed single cell analysis.

Microfluidic PCR of single cells has proven useful for a number of applications[39], especially for studying the gene expression of pluripotent cells for cell therapy[40]. Yet its throughput still needs to be improved, and it is difficult to amplify the genome of a single cell evenly and without bias. Recent contributions have demonstrated a significant improvement in throughput for these systems, performing multiple necessary functions on the same chip and increasing the number of reactions in a practical manner. White et al introduced a digital PCR system, capable of handling ~10^5 PCR reactions at shot noise limited performance[41] (Fig. 1b). Dennis et al introduced a RT-PCR approach capable of performing ~10^4 reactions in parallel[42]. Also, in a recent publication, Gole et al utilized nanoliter wells to provide physical separation of small volumes for unbiased amplification[43]. In addition, rare pathogen detection and quantification in the presence of 100,000 fold excess of other cells has been demonstrated using quantitative PCR (qPCR)[44].

The transcriptome can serve as an important gateway to understanding gene expression and gene-gene interactions. Although the transcriptome of a single cell can be analyzed using current commercial technology, improvements in throughput are necessary for clinical practicality due to high sample numbers[45]. Improvements in microfluidic techniques, such as the addition of individually addressable reaction chambers for parallel processing aid greatly in improving throughput, as demonstrated by learning about the genetic stability of cell populations through the expression of housekeeping genes[46]. The transcriptome can also serve as a marker for diagnostic purposes. Rane et al demonstrated single pathogen detection in an amplification free manner using FRET-labeled peptides to detect pathogenic RNA[47]. Another amplification free method utilized nano sized carrier complexes containing complement fluorescent oligonucleotides for sensing intracellular tumor RNA[48].

Intracellular measurements of single cells can be performed in bulk by using the cell membrane as the compartment. However, in this methodology, measurements are not independent and at least part of the detection system must be present within the cells. A recent method combined the analytical strength of ELISA with a microfluidic system performing all the necessary steps including isolated trapping, washing, and lysis[49]. Using this method, intracellular proteins, secondary messengers, and metabolites can be quantified.

The heterogeneity between single cells was recently investigated, utilizing novel microfluidic approaches that improve throughput or combine multiple operations on the same chip. Wilson et al studied the heterogeneity of stem cell differentiation within embryoid bodies (EB) by dissociating the EBs into single cells and using a microfluidic trapping array[50]. Metto et al studied the heterogeneity of single T-Lymphocyte cells monitoring variations in induced nitric oxide production in an automated fashion, incorporating cell transport, lysis, injection, electrophoretic separation, and fluorescence detection in a single device[51].

Measuring cell deformability can be used for cancer diagnostics[52,53]. It was recently investigated, utilizing optical stretching, a technique that traps and applies mechanical strain with two lasers, to probe the deformability of healthy versus cancer cells for the rapid and early diagnosis of oral squamous carcinoma[54]. In addition, a microfluidic analog of whole-cell micropipette aspiration (MPA) allows for more simple and practical measurement of single cell deformability as compared to MPA[55].

Measuring cytotoxicity is important for profiling immune responses for the development of cancer immunotherapy, however studying cytotoxicity on the single cell level is challenging and calls for high throughput co-incubation systems. Recently, nanowells has been used for co-incubation and analysis of T cell mediated cytotoxicity on tumor cells[31]. Lastly, 2D non-compartmentalized cell patterns have also been used. Hsiao et al immobilized tumor cells with DNA-based linkages for cytotoxicity studies following the addition of PBMCs, antibodies, and a viability probe[29]. The genotoxicity of various nanomaterials on single cells was also studied by using electrostatic interactions to create cell patterns and monitoring DNA diffusion through gel matrixes following nanomaterial exposure[30].

Long term assays requiring incubation

When discussing the incubation of single cells, it is important to distinguish between microfluidic systems that compartmentalize the cells and those that do not as well as their ability to support adherent cells. Chemical compartmentalization allows for the creation of separate microenvironments, eliminating the dependence between samples, making it essential for facilitating parameter analysis on individual cells. It also allows for enhanced system sensitivity by increasing the effective concentration of species in the medium. Compartmentalizing cells using single emulsion water in oil (W/O) droplets is a common and expanding

microfluidic method to chemically isolate single cells. Analyzing cells in flowing droplets has been shown for prolonged cellular processes, such as single cell secretion in agarose droplets coupled to flow cytometry[56]. However, non-immobilized droplet systems make specific cell tracking over time nearly impossible. Stationary culture arrays, which are defined by the geometry of the device[57] or created using other methods[58], allow for simple and practical tracking using time lapse microscopy. For example, Bai et al developed a double droplet trapping and co-incubation system that allowed for the incubation and visualization of quorum sensing between droplets using conventional fluorescent microscopy[59]. Other methods utilize confining geometries such as a mesh grid for on-demand droplet docking into arrays or grids[60,61]. Although, during prolonged incubation and observation, immobilized droplets are prone to evaporation, especially in porous PDMS devices. Different techniques such as water soaking or coverslip immersion can be employed to balance water permeation to and from PDMS devices, as recently demonstrated by the single cell incubation and observation of microalgae for over one month[62]. Another approach was recently demonstrated by the use of double emulsion (W/O/W) droplets, allowing for the large aqueous phase to balance water permeation as well as interface the droplets with common aqueous phase based cell assays[63]. An alternate and novel approach for stationary chemical compartmentalization bypasses the droplet route entirely by utilizing chambers that are chemically isolated with valves creating individually addressable chambers for programmable multistep protocols[64]. Chemically compartmentalized agarose chambers were also used to measure the cell-cell variability of yeast cell transcription response due to pheromone activation[65].

Cellular secretion is an important biological function for the production of therapeutics[7] as well as understanding the immune system[66], angiogenesis[67,68], cancer development[69], and more[70]. Studying cellular secretion at single cell resolution can be an important tool for understanding underlying mechanisms while considering cell heterogeneity and phenotypic variations due to epigenetics and changes in the cellular microenvironment. Analyzing single cell secretion using microfluidics requires chemical compartmentalization of single cells, often in a stationary droplet format, to allow for tracking over prolonged time scales (hours). Microengraving, a powerful and prominent technique for single cell secretion assays, captures the protein secretions of thousands of single cells on a substrate in an array format for further analysis and detection. This technique has been recently used to analyze the heterogeneity of tumor cell secretion behavior[69], study T cell synapse formation with immobilized lipid bilayers with tethered ligands[66], and to screen autoantibodies from autoimmune disease rheumatoid arthritis patients[57] as well as Sjögren's syndrome mice[71]. Sendra et al demonstrated cell retrieval and downstream functionality with microengraving and Yamanaka et al introduced fluorescent cellular barcoding to the microengraving technique, thereby increasing efficiency as well as permitting the quantitative analysis of secretory networks of cell-cell interactions in multicelled wells[72]. Another single cell secretion platform recently enabled the functional screening of up to 300 000 individual hybridoma cell clones in less than one day, an improvement in both throughput and practicality[73].

Non-compartmentalized single cell incubation, useful for growth monitoring or other cell assays, have been recently reported in various works. A method published by Son et al brings the precise (up to 0.01%) mass measurement of single cells using suspended microchannel resonators (SMR) and correlates single cell mass tracking to cell cycle progression[74]. Non-compartmentalized micropatterning can also be a useful tool to study cell-cell contacts and interactions by utilizing adhesive promoting patterns in close proximity. Although the common methodology relies on random cell seeding, microfluidic devices[75] or laser guidance[32] can be integrated to improve seeding efficiency and resolution. Cell-cell interactions between cell pairs have also been studied in non-compartmentalized culture arrays created by dielectrophoresis[76]. Furthermore, the powerful combination of microfluidics, microscopy, and computational tools has allowed for bringing real time external feedback loop systems controlling gene expression from the population level down to the single cell level[77]. Single cell growth tracking has been studied, even with asymmetrically dividing yeast cells[78], permitting single cell culture on the scale of days. Non-compartmentalized single bacteria platforms, useful for many applications relating to the biotechnology industry, have been recently used for determining antibiotic resistance[79] as well as the effects of environmental changes and nutrients on cell growth of industrially relevant bacteria[80].

For many applications, there is a motivation for single cell analysis or incubation with adherent cells, since the majority of cell types are adherent. Adherent cell culture requires special cell compatible substrates for cell attachment or perhaps 3D gels for the most optimal culture conditions as it mimics the growth environment *in vivo*. High throughput single cell containing micro-gels can be created by gel photo-polymerization under a mask containing a repeating micro-pattern[81]. The shapes of the micro-patterns on the mask can be used to manipulate the geometry of the micro-gel. 3D gels are also important for cell migration studies. Single cancer cell migration tracking in gels was studied recently by Nguyen et al, using electrical cell-substrate impedance sensing[82] (Fig. 1c) with an array of microelectrodes, each for every cell, giving single cell data crucial for understanding the kinetics of the process and the heterogeneity of the population. Microengraving can also serve as a non-compartmentalized single adherent cell analysis system as described previously by removing the coverslip.

Recently, a method has been presented to obtain a cell suspension from an adherent cell population while maintaining them in their adhered state by culturing them on fibronectin coated parylene microplates[83], which are bound to a substrate and can be enzymatically detached. This method can theoretically make any microfluidic platform biocompatible with adherent cells, while preventing the trypsinization process which stresses the cells and can induce perturbations in cell physiology.

Conclusions and future directions

Compartmentalization of single cells for single cell analysis enables the creation of many independent experiments, a crucial feature for high-throughput analysis platforms and high sensitivity assays. Compartmentalization over prolonged time periods also allows for studying cell secretion of different cells, an important function for disease pathology, as well as manufacturing of biological therapeutics[7,69]. Although the majority of cell types are adherent, few adherent single cell studies were demonstrated in the field of microfluidics, most likely due to the lack of compatible systems. In addition, we further note the increased scarcity of platforms that can support the analysis or incubation of adherent cells that are also compartmentalized. In order to achieve this, not only do cells need to be compartmentalized, but the compartments

themselves should be static and interface with or include a substrate that is able to support cell adhesion. In general, aside from the advantages and capabilities mentioned here, in order for microfluidic SCA platforms to be integrated to the common biological laboratory with untrained personnel, ease of operation is critical, and perhaps can be the focus of future efforts.

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Notes and references

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