Recent advances in microfluidic techniques for single-cell biophysical characterization

Yi Zheng, John Nguyen, Yuan Wei and Yu Sun*

Biophysical (mechanical and electrical) properties of living cells have been proven to play important roles in the regulation of various biological activities at the molecular and cellular level, and can serve as promising label-free markers of cells’ physiological states. In the past two decades, a number of research tools have been developed for understanding the association between the biophysical property changes of biological cells and human diseases; however, technical challenges of realizing high-throughput, robust and easy-to-perform measurements on single-cell biophysical properties have yet to be solved. In this paper, we review emerging tools enabled by microfluidic technologies for single-cell biophysical characterization. Different techniques are compared. The technical details, advantages, and limitations of various microfluidic devices are discussed.

Introduction

The cell, the basic functional unit of living organisms, maintains and senses the physiological environment within the organism both chemically and physically.1–4 The unique biochemical and biophysical properties enable a cell to fulfill its specific functions and adapt to its surrounding environment. Physiological changes within the cells are accompanied by chemical and physical modifications and reorganization. Thus, pathological cells can be identified biochemically and/or biophysically. Biochemical properties of pathological cells have been under intensive study, and many biochemical markers have been developed to identify target cells out of a heterogeneous population.5,6 The biophysical properties of cells also play important roles in various biological processes and are involved in the regulation of gene expression, differentiation, migration, and metabolic activities.2,7 However, although research on the physical properties of cells has provided strong evidence about the capability of physical properties as potential markers for identifying cell types, most biophysics research was limited to proof-of-concept demonstrations. The lack of clinical relevance is due to the very low measurement throughput and tedious operation procedures of conventional techniques for measuring the biophysical properties of cells.4,7–9 Compared to conventional techniques, the advantages of small sample volume, integration capability, biocompatibility and fast response make microfluidic technologies attractive for studying cells. Recent decades have witnessed significant advances of microfluidic technologies for biochemical characterization of cells.10,11 Microfluidics is extending its way into the characterization of single-cell biophysical properties.12–14

In this review, we summarize existing microfluidic technologies for characterizing biophysical properties of individual cells (i.e., mechanical and electrical properties). Within each section, we first provide an introduction of a technique. We then classify various techniques based on their working mechanisms and discuss their advantages and limitations. Finally, we present perspectives on the challenges for diagnostic applications and future directions of microfluidic technologies for single-cell biophysical characterization. This review is focused on discussing techniques for characterizing single-cell mechanical and electrical properties. Microfluidic techniques for studying the roles of mechanical and electrical stimuli in the regulation of differentiation, migration, and metabolic activities have been reviewed elsewhere.3,4,13

Mechanical characterization techniques

The association between cell deformability and human diseases has been of interest since the 1960s.13,16 The deformability of nucleated cells is determined by the membrane, the cytoskeletal network (actin filaments, intermediate filaments, and microtubules), and its interaction with the nucleus, while the deformability of red blood cells (RBCs) is determined by the membrane skeleton network and the interaction between the membrane skeleton and membrane integral proteins.4,17,18 Physiological and pathological changes

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can alter the cytoskeleton composition, reorganize the network structure, and change the protein density. As a result, cell deformability can be used as an intrinsic marker for identifying pathological conditions.\(^4,19,20\) For example, deformability is known to play a crucial role in the mobility of cancerous cells;\(^4,21\) and a decrease in RBC deformability has been proven to be relevant in several human diseases (see Table 1).

Compared to existing experimental tools, such as atomic force microscopy, micropipette aspiration, optical tweezers, and magnetic tweezers, which are all difficult to use and have a low testing speed, microfluidics offers the potential for high-throughput mechanical measurement of single cells.\(^4,10,12,13\) For characterizing the mechanical properties of a cell, the cell must be deformed. Thus, we classify microfluidic technologies for cell deformability measurements according to the mechanical stimuli used to deform the cell. Table 2 summarizes these techniques and their working mechanisms, cell samples, key technologies and observations, and reported throughput. For those where throughput was not reported, the total number of tested cells is listed in Table 2 in order to provide a sense of the devices' throughput.

**Structure-induced deformation (constriction channels)**

Constriction channels, which are marginally smaller than the diameters of tested cells, provide an efficient method to generate mechanical stimuli. Cells driven through a constriction channel are squeezed by the walls of the constriction channel. Multiple parameters, such as transit time, elongation and recovery time, in association with cell deformability can be quantified. Moreover, constriction channels can be easily fabricated with standard microfabrication techniques and are able to provide an environment to mimic in vivo capillaries. With the use of high-speed imaging or electrical impedance measurements, constriction-channel devices are capable of achieving a higher throughput than most other deformability measurement approaches. Due to these merits, constriction channels have been used to measure the deformability of RBCs,\(^22,23,39–44\) leukocytes\(^45\) and cancer cells.\(^46,47\)

Due to the human capillary-like environment and the physiological relevance of RBC deformability, RBCs are mostly studied in the majority of existing constriction channel-based devices. The first demonstration of microfluidic constriction channels for RBC deformability characterization was reported in 2003.\(^22\) In this study, constriction channels with various diameters were used to study the deformability changes between healthy RBCs and malaria parasite-infected RBCs at different stages (early ring stage, early trophozoite, late trophozoite, and schizont). They found that the deformability of malaria-infected RBCs decreases as the parasite progresses from the early ring stage to a schizont, while healthy RBCs are exceptionally deformable and are even able to travel through the constriction channels blocked by infected RBCs. In addition to RBC deformability, white blood cell (WBC) deformability was also studied using microfluidic constriction channels. Rosenbluth et al. demonstrated the clinical relevance of their microfluidic constriction channel device in sepsis and leukostasis.\(^45\) The reported device consists of a network of 64 constriction channels. High-speed imaging was used for measuring cell transit time. They used patient samples to show that cell transit time increased for diseased samples compared to control samples (Fig. 1(a)). In another study, breast cancer cell lines (MCF-7 and MCF-10A) were flown through a constriction channel to distinguish non-malignant and malignant cells.\(^46\) According to the reported results, transit velocity is not significantly affected by cell types, and the transit time difference is mainly determined by the entry time. Different cell types can be distinguished on the basis of scatter plots of cell volume and entry time.

Besides imaging, microfluidic constriction channels can also be used together with other measurement techniques to achieve multiple parameter measurements for cell type classification. We recently developed a microfluidic device (Fig. 1(b)), which combines a constriction channel and impedance measurements.\(^49\) Detection involves only electrical signals; hence, it enables a throughput higher than 100 cells s\(^{-1}\). The multiple parameters quantified as mechanical and electrical signatures include transit time, impedance amplitude ratio, and impedance phase increase. Histograms compiled from 84 073 adult RBCs and 82 253 neonatal RBCs reveal different biophysical properties across samples and between the adult and neonatal RBC populations. Bow et al. demonstrated a deformability-based RBC testing device combining constriction channels and fluorescence measurement. They showed a population-based correlation between biochemical properties, such as cell surface markers, and mechanical deformability.\(^21\) They also showed experimentally that the entrance geometry of the constriction channel has a significant impact on RBC transit time, and developed a dissipative particle dynamics model to interpret the parasitic effect on RBC deformability. An optical stretcher was also introduced to enhance the sensitivity and reliability of a constriction channel-based microfluidic device.\(^40\) Scatter plots

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<th>Possible genetic and molecular cause</th>
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<td>Malaria</td>
<td>Interruption of cytoskeleton network by parasite protein</td>
<td>17, 22–25</td>
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<td>Hereditary spherocytosis</td>
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<td>17, 26–28</td>
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<td>Sepsis</td>
<td>Altered spectrin interaction</td>
<td>31–33</td>
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<td>Diabetes</td>
<td>Not clear</td>
<td>34–36</td>
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<td>Stored RBCs</td>
<td>Depletion of ATP and Nitric oxide</td>
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Table 2 Microfluidic devices for single-cell mechanical characterization

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<td>Constriction channels of different cross-sectional areas</td>
<td>Malaria infected RBCs</td>
<td>Uninfected RBCs can readily travel through the constriction channel. RBCs become less deformable as the stage of malaria infection progresses.</td>
<td>N/A</td>
<td>22</td>
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<td>Cuneiform constriction channel array + fluorescence</td>
<td>Malaria infected RBCs</td>
<td>Entrance geometry of the constriction channel has a significant impact on RBC transit time. Deformability measurements of ring stage <em>P. falciparum</em>-infected and uninfected RBCs show significant overlap.</td>
<td>$\sim 100$ cells min$^{-1}$</td>
<td>23</td>
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<tr>
<td><strong>Constriction channel + impedance measurement</strong></td>
<td>Adult and neonatal RBCs</td>
<td>Characterization of multiple parameters (transit time, impedance amplitude and impedance phase) improves the classification accuracy.</td>
<td>$100$–$150$ cells s$^{-1}$</td>
<td>39</td>
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<td>Constriction channel + optical pressure</td>
<td>RBCs from healthy donors and leukemia patients</td>
<td>Recovery time of RBCs from healthy donors and leukemia patients was found to be significantly different.</td>
<td>$N = 140$</td>
<td>40</td>
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<td><strong>Constriction channel array</strong></td>
<td>Blood cells from AML and ALL patients, and HL-60 cell lines.</td>
<td>Transit time of WBCs from AML patients with leukostasis symptoms is significantly higher than the WBCs from AML patients without leukostasis symptoms and ALL patients.</td>
<td>$50$–$100$ cells min$^{-1}$</td>
<td>45</td>
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<tr>
<td>Constriction channel</td>
<td>MCF-7 and MCF-10A</td>
<td>The different transit time is mainly contributed by the longer entry time of MCF-7.</td>
<td>$N \approx 100$</td>
<td>46</td>
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<td>Constriction channel + impedance measurement</td>
<td>MC-3T3 and MLO-Y4; EMT6 and EMT6/AR1.0</td>
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<td>$\sim 1$ cells s$^{-1}$</td>
<td>47</td>
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<td>Constriction channel + volume measurement</td>
<td>HeLa</td>
<td>Controlled Hela cells have longer transit time than Hela cells treated with latrunculin A and cytochalasin B. MCF-7 and MCF-10A can be distinguished on the basis of transit time.</td>
<td>$800$ cells min$^{-1}$</td>
<td>48</td>
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<td><strong>Constriction channel + deformable membrane</strong></td>
<td>MCF-7 and MCF-10A</td>
<td>The prediction of disease states in patients with cancer and immune activation achieved a sensitivity of $91%$ and a specificity of $86%$. Effects of diamide and glutaraldehyde on RBC dynamics were examined by visual observation of cells when flowing through a microchannel.</td>
<td>$N = 150$–$200$</td>
<td>49</td>
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<tr>
<td>Fluid-induced</td>
<td>Leukocytes and malignant cells in pleural effusions; pluripotent stem cells</td>
<td>The results prove that hyperbolic shape is more efficient in deforming RBCs.</td>
<td>$\sim 2000$ cells s$^{-1}$</td>
<td>19</td>
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<tr>
<td>Inertial focusing + hydrodynamic stretching</td>
<td>Blood cells from AML and ALL patients, and HL-60 cell lines.</td>
<td>The prediction of disease states in patients with cancer and immune activation achieved a sensitivity of $91%$ and a specificity of $86%$. Effects of diamide and glutaraldehyde on RBC dynamics were examined by visual observation of cells when flowing through a microchannel.</td>
<td>N/A</td>
<td>50</td>
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<td>Straight microchannel</td>
<td>RBCs and fixed RBCs</td>
<td>Time taken for complete electroporation-induced lysis can be correlated to the defects in the cytoskeleton network.</td>
<td>N/A</td>
<td>61</td>
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<tr>
<td><strong>Hyperbolic converging microchannel</strong></td>
<td>RBCs</td>
<td>The results prove that hyperbolic shape is more efficient in deforming RBCs.</td>
<td>N/A</td>
<td>51</td>
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<td>Shear stress + resistance measuring</td>
<td>RBCs and fixed RBCs</td>
<td>The resistance signal generated by integrated electrodes is correlated to the deformation of RBCs.</td>
<td>$N = 72$</td>
<td>83</td>
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<td><strong>Electroporation-induced</strong></td>
<td>Microfluidic channel + high electrical field</td>
<td>Controlled mouse RBCs, mouse RBCs with deficiencies of ankyrin, $\beta$-adducin and Tmod1</td>
<td>More malignant and metastatic cell types exhibit more significant swelling.</td>
<td>$5$ cells s$^{-1}$</td>
</tr>
<tr>
<td>Microfluidic channel + high electrical field</td>
<td>MCF-10A and MCF-7</td>
<td>Time taken for complete electroporation-induced lysis can be correlated to the defects in the cytoskeleton network.</td>
<td>$2000$–$3000$ cells/sample</td>
<td>63</td>
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<tr>
<td><strong>Optical stretcher</strong></td>
<td>RBCs; BALB 3T3</td>
<td>Optical deformability can be used to differentiate cells by measuring the differences in elastic response.</td>
<td>N/A</td>
<td>67</td>
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<tr>
<td>Microfluidic channel + laser beam</td>
<td>MCF-10A, MCF-7 and modMCF-7; MDA-MB-231; BALB/3T3 and SW-T2</td>
<td>Cancer cell lines are significantly more deformable than normal cells.</td>
<td>N/A</td>
<td>70</td>
</tr>
<tr>
<td>Microfluidic channel + laser beam + microcouplers</td>
<td>RBCs</td>
<td>A two-axis active microcoupler capable of generating substantial displacement for accurate alignment of buried optical fibers was developed.</td>
<td>N/A</td>
<td>71</td>
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Compiled from transit time, elongation, and recovery time measured by this device proved effective for the discrimination of RBCs from normal donors and leukemia patients.

Other applications of microfluidic constriction channels include the use of wedge-shaped constriction channels to measure the surface area and volume of a large population of RBCs, and a microfluidic manometer to measure the pressure drop due to the presence of a cell in the constriction region, which correlates with the stiffness of the cell, by observing the displacement of the downstream fluid-fluid interface. Despite the advantages of the constriction channel technique, cell volume and adhesion between the cell membrane and channel walls are coupled with cell deformability. Consequently, a longer transit time does not necessarily mean lower deformability since larger and stickier cells can also lead to a longer transit time. Efforts have been made to take the cell volume/size effect into account. For example, Adamo et al. reported a microfluidic device for probing both cell volume and transit time. Comparisons of cell transit time were made among cells with a similar volume. They demonstrated that Hela cells in the control group have a longer transit time than Hela cells treated with latrunculin A and cytochalasin B. The size of the constriction channel can also be adjusted on demand according to targeted cell size and cytochalasin B. The size of the microfluidic constriction channel is smaller than the diameter of the targeted cells, the channel is susceptible to clogging. Using an array of constriction channels appears to be a potential solution to the clogging issue.

**Fluid-induced deformation**

RBCs are highly deformable and can easily deform under fluid shear stress inside blood vessels. Having micrometer dimensions, which are comparable with in vivo capillaries, microfluidic channels provide an ideal tool for investigating RBC deformability. Compared to constriction channels, the microfluidic channels used to generate shear stress are larger than RBCs. Thus, RBCs are deformed by fluid shear stress instead of the channel’s confinement structures. The deformation index (DI) or stretch ratio quantified via high-speed imaging has been used as a measure of RBC deformability. Forsyth et al. studied the deformability and dynamic behavior of chemically “stiffened” RBCs using a simple straight microfluidic channel, revealing three different types of motion due to the increased shear rate in the microfluidic channel: stretching, tumbling, and recoiling. Besides straight channels, a hyperbolic converging microchannel was also developed for assessing RBC deformability by measuring extensional flow-induced deformation. The results confirmed that extensional flow is more efficient in causing RBC deformation. Shear stress generated in microfluidic channels was also used to measure the dynamics of shear-induced adenosine triphosphate (ATP) release from RBCs. In ref. 52, RBCs were driven through a microfluidic channel with a cross-sectional area of 20 × 20 µm, while the amount of ATP released was measured by counting the photons emitted from a standard luciferase-ATP bioluminescent reaction. Cell deformation and dynamics were quantified simultaneously using high-speed imaging.

Although shear stress proves effective for investigating RBC deformability, the low magnitude of shear stress is typically not able to deform other types of cells. Gossett et al. recently reported a hydrodynamic-stretching microfluidic device for identifying malignant cells in a human pleural fluid sample with a measurement speed of ~2000 cells s⁻¹ (see Fig. 1(c)). Cells are focused towards a narrow streamline near the center of the microfluidic channel and delivered to a junction of two orthogonal channels at a high flow rate, where the cells undergo mechanical stretching. Meanwhile, cell deformations are captured using a high-speed camera and images are then analyzed to extract the cell volume and deformation index (DI). The results revealed that cancerous cells in pleural fluid have

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<th>Throughput⁶</th>
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<td>DEP-induced</td>
<td>Microfluidic channel + laser beam, Microfabricated electrodes</td>
<td>Healthy and cancerous oral squamous cells, CHO-K1 and U937</td>
<td>Cancer cells showed a higher mean deformability and increased variance. Cell deformation was interpreted into mechanical properties using an analytical model.</td>
<td>N = 200–300</td>
</tr>
<tr>
<td>Microfabricated electrodes</td>
<td>SiHa and ME180</td>
<td>A FEM model was developed to extract the Young’s modulus of cells. RBCs from patients with spherocytosis are less deformable than RBCs of healthy volunteers.</td>
<td>N = 200–300 for each cell type</td>
<td>79</td>
</tr>
<tr>
<td>ITO electrodes + microchamber array</td>
<td>Healthy and spherocytosis RBCs</td>
<td></td>
<td></td>
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<tr>
<td>Aspiration-induced</td>
<td>Funnel chain + pressure attenuator, Microfluidic pipette + impedance measurement</td>
<td>Healthy and malaria RBCs, MC-3T3</td>
<td>Malaria infected RBCs were proven less deformable than normal RBCs. MC-3T3 was characterized by impedance spectroscopy and micropipette aspiration.</td>
<td>N = ~100 for each cell type</td>
</tr>
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⁶ Throughput: For those references that explicitly reported speed/throughput, speed/throughput is listed directly in the table. For those references where speed/throughput was not explicitly reported, we calculated speed/throughput using the data plots (e.g., histograms) presented in those references.
larger volumes and are more deformable (higher DI values) than benign cells. This approach achieved both a high testing speed and larger deformation of tested cells. The authors predicted disease states in patients with cancer and immune activation with a sensitivity of 91% and a specificity of 86%.

In fluid-induced deformation-based microfluidic devices, channel dimensions are larger than cell diameters. DI is used as the deformability indicator and is not affected by the adhesion between cell membrane and channel walls (vs. constriction channel). However, since cells of different sizes may experience different forces due to non-uniformity in the shear stress and hydrodynamic pressure within the microchannel, DI is still associated with cell volume. More specifically, shear stress is highest near channel walls and is almost zero in the center of the channel. As a result, RBCs with larger volumes experience higher shear stress on the edges causing larger deformation compared to cells with smaller volumes. Within the hydrodynamic-stretching microfluidic device, the highest compressive pressure appears at the center of the junction where the cells are stretched, which causes...
cells with different volumes or shapes to be exposed under a different pressure environment. To address this issue, cell volume is measured as an independent parameter and used as a reference for cell-type classification.19

A limitation of fluid-induced deformation-based microfluidic devices is the use of high-speed imaging (tens of kHz) for measuring DI. High-speed cameras are often costly and must be used on a microscope, making the overall microfluidic system bulky. Furthermore, since real-time image data transfer from a high-speed camera to the computer hard drive is technically difficult to achieve, images are stored in the camera’s on-board memory for later off-line transfer. Since state-of-the-art high-speed cameras typically have only a few GB of on-board memory, after recording for a few seconds, the experiment must be stopped for image data transfer. Processing massive amounts of image data also costs tremendous computational effort and time. Advances in high-speed cameras and hardware-based image processing techniques, such as FPGA-based techniques, will possibly be able to solve the data transfer problem. Additionally, opto-microfluidics, microfluidic devices integrated with optical components, also seems a promising way to tackle this problem. Integrating on-chip lenses and CMOS chips35-56 can achieve real-time performance and in the meantime, eliminate the need for the use of bulky microscopes.

Electroporation-induced deformation

Electroporation is a technique used for introducing foreign molecules, such as DNA and proteins, into cells. The concept of electroporation capitalizes on the relatively weak nature of the phospholipid bilayer’s hydrophobic/hydrophilic interactions and its ability to spontaneously reassemble after a disturbance.57 Thus, a voltage shock can disrupt areas of the membrane temporarily, allowing polar molecules to pass. The membrane then can re-seal and leave the cell intact.57-59

Swelling or expansion in cell size accompanies the cell’s electrical property changes,60 which is caused by the influx of molecules through the open pores in the cell membrane (vs. dielectric force in DEP-induced deformation). Recently, a few studies correlated electroporation-induced cell deformation and lysis to cell deformability changes. Bao et al.61 developed microfluidic electroporative flow cytometry to study the deformability of single cells. A constant DC voltage was established across the microchannel, which concentrated the electric field to yield repeatable cell exposure to uniform electrical fields. When cells were flown through the microchannel, the swelling of cells was recorded by imaging and quantified as a deformability indicator. Deformability changes of breast cancer cell lines61 and the expansion of the nuclei of circulating tumor cells (CTCs) were tested using the system.62

The capability to detect RBCs with deficiencies of the cytoskeletal protein network was also demonstrated (see Fig. 1(d)).61 The achieved throughput was about 5 cells s⁻¹.

It is notable that electroporation efficiency is also dependent on cell volume. Under the same uniform electrical field, the effective voltage across the cell membrane is a function of cell diameter.61 Hence, cells with larger diameters are exposed to higher voltages and are more easily porated. In addition, the plasma membrane of different cell types (e.g., metastatic tumor cells vs. non-metastatic tumor cells) can possess different poration properties. In other words, some cell types may be more susceptible to electrical fields than others. Thus, based on electroporation-induced expansion or swelling alone, it can be inaccurate to conclude that cell deformation is caused by cell deformability rather than the membrane’s poration susceptibility to electrical voltages.

Optical stretcher

Optical trapping was discovered in 197065 when radiation pressure from laser light was found to be able to accelerate and trap micron-sized dielectric particles. Light interacts with a particle by imparting some of its momentum onto it, thus exerting a force on the particle. If the particle is not centered on the optical axis of the beam, a restoring force is exerted on the particle to keep it on the optical axis. Optical stretchers utilize two slightly divergent Gaussian beams to trap an object in the middle. This concept was used to deform and measure the stress profiles of erythrocytes, which led to the development of the first optical stretcher device reported in 2000.66

The setup was then demonstrated to stretch BALB 3T3 fibroblasts and measure their viscoelastic properties.67

A typical optical stretcher system consists of a microchannel for loading cells into the testing region and two laser fibers located on the sides of the passageway (see Fig. 2(a)).68 Cells flowing through the microfluidic channels are serially trapped and deformed with the two counter propagating divergent laser beams.69,70 For trapping and stretching cells in the optical stretcher, the alignment of the fibers is crucial. In order to improve alignment, a platform capable of adjusting fiber positions was developed71 using pneumatically driven manipulators. The femtosecond laser technique has been utilized for the fabrication of optical stretcher-based microfluidic devices.72,73 By direct writing of both optical waveguides and microfluidic channels, the alignment problem can be mitigated significantly. The deformability of human cancer cell lines,69,70 red blood cells71 and patient oral squamous cells74 was characterized using optical stretchers.

DEP-induced deformation

When polarized in an electric field, biological cells will experience a dielectric force, which is well known as a DEP force.75,76 Electro-deformation devices utilize the DEP force for trapping and generating mechanical stimuli to quantify the deformability of individual cells.77,78 The Young’s modulus of tested cells can be extracted with either analytical models or numerical simulation. For improving the throughput of electro-deformation devices, a single-cell microchannel array device79 was proposed. The device is able to trap individual RBCs in a large array of micro-wells integrated with DEP electrodes. ITO electrodes also allow the correlation of RBC deformation with cell surface and cytosolic characteristics (Fig. 2(b)). However, due to the time-consuming procedure of cell trapping, the overall throughput of this device is still rather limited. On the other hand, the complex physical phenomena involved in electro-deformation and unknown cell electrical properties pose difficulties in extracting forces experienced by an electro-deformed cell.75,77,78 Since the DEP force is determined by the dielectrical properties of cells, DEP-
based techniques can also be used to electrically characterize cells, which is discussed in the electrorotation section under electrical characterization.

**Aspiration-induced deformation**

Pipette aspiration is a conventional technique for studying the mechanical properties of single cells. The mathematical models in conventional pipette aspiration have been adopted in microfluidic characterization of cells. Fig. 2(c) shows a microfluidic pipette aspiration device. Single cells are infused into a microfluidic channel and deformed through a series of funnel-shaped constrictions. Malaria-infected RBCs were tested using both membrane cortical tension and threshold pressure as readouts. We developed a microfluidic device for single-cell electrical and mechanical characterization using impedance spectroscopy and micropipette aspiration. Cellular deformation was recorded as a function of increasing pressure, while cellular impedance was measured via two Ag/AgCl electrodes inserted into the culture medium. With pipette aspiration and equivalent circuit models, both mechanical properties and dielectric properties of single cells were quantified. In addition to throughput limitation, the rectangle-like cross-section in microfluidic pipette aspiration devices can cause concerns about the validity of applying conventional pipette aspiration models.

**Electrical characterization techniques**

Besides mechanical deformability, electrical properties of cells are also important physical properties, serving as the basis of counting, trapping, focusing, separating, and the characterization of single cells. Early work on cell electrical measurements dates back to the 1910s, when approximate hemoglobin conductivity inside RBCs was reported. The single shell model proposed by Pauly and Schwan in the 1950s laid...
the foundation for interpreting the electrical properties of cells, where the cell is modeled as a spherical cytoplasm surrounded by a thin dielectric membrane.\textsuperscript{89,90} Generally, the electrical properties of a plasma membrane are affected by the membrane morphology, lipid bilayer composition and thickness, and embedded proteins.\textsuperscript{91–93} Electrical properties of the cytoplasm are influenced by the intracellular structures and physiological conditions (e.g., nucleus-to-cytoplasm ratio and ion concentrations inside the cell).\textsuperscript{94–96} Early techniques were only capable of measuring the average electrical properties of a cell population. The patch-clamp technique, developed in 1976 is capable of electrical characterization of single cells.\textsuperscript{97} However, a patch-clamp typically takes tens of minutes to test one cell. Because of the capability of manipulating microscaled objects and integrating microelectrodes on-chip, microfluidic techniques have gained momentum in single-cell electrical characterization in recent years. Table 3 summaries and classifies microfluidic techniques for the electrical characterization of single cells.

**Microfluidic Coulter counter**

The Coulter counter monitors the DC resistance of a small orifice as microparticles pass through. Since the cell membrane acts as an insulating layer at DC, the presence of the cell alters the resistance of the orifice by replacing the conductive liquid. Well established models are available for correlating DC resistance changes to cell volumes. Several designs have recently been proposed to improve the microfluidic Coulter counter’s performance. For instance, two-dimensional sheath flow focusing was demonstrated to overcome the clogging issue;\textsuperscript{98} and throughput of microfluidic Coulter counters was improved using multiple-orifice designs.\textsuperscript{99}

One challenge for microfluidic Coulter counters lies in the selection of the electrode material. Ag/AgCl non-polarizable electrodes, which work well in the conventional Coulter counter, are an ideal choice. Nonetheless, integrating Ag/AgCl electrodes into microfluidic channels is complex, and Ag/AgCl electrodes have a limited lifetime.\textsuperscript{100,101} Although other metal electrodes can be more readily integrated into microfluidic channels, the electrical double layers formed between the interface of electrodes and the liquid, which are mainly capacitive, pose difficulties in applying DC signals. Methods for minimizing the electrical double layer effect include the modification of the electrode surface roughness in order to increase the surface area\textsuperscript{102} and the utilization of polyelectrolytic salt bridges (PSBEs)\textsuperscript{103} or polyelectrolyte gel electrodes (PGEs).\textsuperscript{104} Most recently, a DC impedance-based microcytometer device integrating PGEs was reported for CTC cell detection. Sheath flow is used for focusing cells and preventing cell adhesion to chambers and channels (see Fig. 3(a)). CTCs were successfully detected in blood samples from breast cancer patients.\textsuperscript{105} Besides the electrode design that demands special design consideration, the model used in the conventional Coulter counter to calculate particle volumes is not directly applicable to microfluidic Coulter counter devices, due to the different electrode configuration and channel geometry.\textsuperscript{106,107} A microfluidic Coulter counter is limited to counting and sizing single cells, but incapable of characterizing their electrical properties.

**Electrorotation**

When a cell/particle is placed in a non-uniform electrical field, a force is exerted on the induced dipole and can cause the cell to either move (DEP) or rotate (ROT).\textsuperscript{75,108} Maxwell’s mixture theory is commonly used to interpret DEP and ROT data, for associating the complex permittivity of the suspension to the complex permittivity of the particle/cell.\textsuperscript{75,108} The DEP force and ROT torque are proportional to the real and imaginary parts of the Clausius–Mossotti factor, respectively.\textsuperscript{7} Although DEP and ROT are closely related, ROT is more suitable for single-cell electrical characterization since in ROT, the cells are only rotated at a certain position in the electric field. Thus, the amplitude of the electric field remains unchanged, which is suitable for fitting the rotation spectra to determine intrinsic electrical properties of single cells (e.g., specific membrane capacitance, cytoplasm conductivity and cytoplasm permittivity). Differently, forces in DEP are determined by the electric field gradient. A constant electric field gradient is technically challenging to achieve. Furthermore, the rotation rate is only determined by the rotation force (constant as a cell rotates) and the viscosity of the suspension medium in ROT, which can be readily measured, whereas the DEP force (varying as a cell moves) is difficult to quantify. DEP was demonstrated to electrically characterize cells by curve fitting of the Clausius–Mossotti factor spectra or cell count spectra. However, since the spectra are not from the measurements of the same cells, only average electrical properties of a cell population can be obtained.\textsuperscript{109–111} More details on DEP and ROT models can be found in other reviews.\textsuperscript{9,112}

The most popular ROT setup uses four electrodes connected to sine waves in phase quadrature.\textsuperscript{91,113} Cells are placed in the center of the four electrodes (e.g., using laser tweezers). Rotation spectra are obtained by measuring the rotation speed at each frequency over a frequency range of 1 kHz to around 100 MHz. By fitting the rotation spectra, specific membrane capacitance, cytoplasm conductivity and cytoplasm permittivity values are determined.\textsuperscript{92,114,115} Fig. 3(b) illustrates the working mechanism of a microfluidic electro-rotation device.\textsuperscript{108} The ROT technique has been used to test a number of cell types, such as white blood cells and human cancer cells.\textsuperscript{91,92,114–116} Electrorotation is a slow technique. It takes approximately 30 min to test a single cell.\textsuperscript{113,117} It is also difficult to achieve efficient rotation in a high conductivity physiological buffer. Hence, electrical properties of the tested cell may have already been altered when immersed in the low conductivity sucrose buffer. Nevertheless, electrorotation is the only method capable of extracting inherent electrical properties of cells, such as membrane permittivity and cytoplasm conductivity. The methodology and electrical models can be instrumental for the development of new techniques for higher throughput.
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*Throughput: For those references that explicitly reported speed/throughput, speed/throughput is listed directly in the table. For those references where speed/throughput was not explicitly reported, we calculated speed/throughput using the data plots (e.g., histograms) presented in those references.*
Microelectrical impedance spectroscopy (μ-EIS)

Microelectrical impedance spectroscopy (μ-EIS) is a technique wherein a frequency-dependent excitation signal is applied across a trapped cell to measure the corresponding current response. Various cell trapping mechanisms incorporated with microfabricated electrodes have been proposed, such as hydrodynamic traps, negative pressure traps, and DEP traps.

Jang et al. developed a microfluidic device which utilizes micropillars within a microfluidic channel to physically capture and measure the impedance of a single human cervical epithelioid carcinoma (HeLa) cell using EIS. Hydralic trapping devices were also demonstrated where impedance measurements were accomplished by recording the current from two electrode pairs, one empty (reference) and one containing a cell. The effect of surfactant and bacterial pore-forming toxins on HeLa cells was monitored continuously. To minimize current leakage, caused by the current undesirably obviating the targeted cell through the low resistivity medium, Cho et al. developed an array of planar micro-holes for positioning cells and forming direct contact between cells and electrodes (see Fig. 3(c)).

Li et al. used a vertical sub-micrometer opening with embedded recording electrodes. The device was capable of reducing the serial resistance, while keeping the seal resistance for high sensitivity measurements. Since single cells can be cultured right on the vertical holes, the capability of monitoring the dynamic changes of single cell electrical properties over a period of time is a merit of the vertical hole-based technique.
There are two major limitations of the μ-EIS technique. Firstly, since the trapping and releasing process is time consuming, and the measurement of impedance spectra also takes time, the throughput of μ-EIS is low. Only tens of cells were tested in reported studies. Secondly, although the capacitance and resistance of tested cells can be determined by interpreting impedance spectra with electrical models, these parameters are strongly affected by electrode size, the cell trapping mechanism, cell volume and the interaction with other cells (vs. size-independent parameters, such as specific membrane capacitance and cytoplasm conductivity).

Impedance flow cytometry (IFC)

Although the Coulter counter has become a widely used technique in clinical instruments (e.g., hematology analyzers), the conventional Coulter counter is only capable of classifying cell types that have distinct volume differences. Some of the most recent commercial hematology analyzers adopted both DC and RF measurements. The ratio of the RF signal to the DC signal is defined as opacity, which is used as a volume independent electrical signature of the tested cells. Along with the DC signal (volume signature), a WBC differentials count can be achieved. This technique was recently demonstrated on microfluidic devices. In addition to WBC count, microfluidic flow cytometry has also been used for analyzing a variety of particles.126–130

An impedance flow cytometry design using coplanar electrodes was demonstrated by Gawad et al.126 As shown in Fig. 4(a), three microfabricated electrodes were integrated on the bottom of the microfluidic channel. When microparticles were flowing through the detection area, the impedance change was recorded differentially. The non-uniform electrical field distribution in the channel, resulting in positional variations of tested cells, may undesirably affect impedance measurement. To overcome this issue, parallel facing electrodes were developed (see Fig. 4(b)).127 When a cell is present in between one pair of electrodes, the other pair is used as a reference, allowing the differential measurement of electrical signals. The device was demonstrated to be capable of detecting electrical property differences between normal RBCs and glutaraldehyde-fixed RBCs.

Holmes et al. incorporated a fluorescence measurement unit on their microfluidic flow cytometry device.129 This device design enabled direct correlation of impedance signals from individual cells with their biochemical phenotypes. A 3-part WBC differential count of human blood was well achieved. The same group more recently developed a microfluidic impedance flow cytometry device integrated with RBC lysis and a hemoglobin concentration measurement unit.131,132 Besides counting blood cells, microfluidic impedance flow cytometry has also found applications in other areas, such as the measurement of cell viability and physiological differences of...
More details on impedance flow cytometry can be found elsewhere.\textsuperscript{112,130} In addition to coplanar and parallel facing electrode designs, Mernier \textit{et al.} demonstrated “liquid electrodes” to discriminate living and dead yeast cells (see Fig. 4(c)). The larger electrodes recessed in lateral channels allow measurements at low frequencies (down to 1 kHz), and the same “liquid electrodes” can also be used for DEP focussing of the particles.\textsuperscript{133} Recently, the concept of label-free electrophysiological cytometry was reported. This technique utilizes the nature of electrically-excitable cells (upon activation by sufficient transmembrane electric fields, different cell types generate different extracellular field potential signals) to distinguish different cell types. As shown in Fig. 4(d), the device is integrated with electrodes for both electrical stimulation and recording of extracellular field potential signals from suspended cells in flow.\textsuperscript{134} Undifferentiated human-induced pluripotent stem cells (iPSC) and iPSC-derived cardiomyocyte (iPSC-CM) cells were distinguished.

**Other physical characterization techniques**

While clinical applications have been mostly driving the development of microfluidic tools for single-cell biophysical characterization, these new tools are also enabling biologists to gain insights into the cell development process. For instance, the microfluidic device reported in ref. 140 for the first time enabled the measurement of the mass of microparticles. When particles suspended in liquid flow through nanomechanical resonators, the resulting frequency shift is used to determine the mass of the particles with extraordinary sensitivity ($10^{-21}$ g). Combined with microfluidic control, the ‘instantaneous’ growth rates of individual cells were determined,\textsuperscript{141} and it was found that heavier cells grew faster than lighter cells. Using this technology, the density of single living cells was also measured.\textsuperscript{33} As shown in Fig. 5(a), the suspended microfluidic resonator consists of a silicon cantilever and an embedded microfluidic channel. When cells flow through the microfluidic channel, the changes in resonance frequency are proportional to the buoyant mass of the cell. In order to measure the density of the cell, the
buoyant mass of the targeted single cells was recorded in two fluids of different densities. The absolute mass and density were then determined and were used to distinguish malaria-infected RBCs, RBCs from patients with sickle cell disease and thalassemia, and drug-treated leukemia cells. The microfluidic system has a testing speed of \( \sim 500 \text{ cells h}^{-1} \).

Compressive forces can be applied directly to cells through a mechanism similar to bulging elastomeric valves.\(^{142}\) In general, such devices consist of multiple layers and a thin elastomeric membrane separating the flow and control channels. The cells within the flow channels are compressed when a pneumatic pressure is applied to deflect the thin membrane. Microfluidic devices based on the compression have been used to monitor cell viability and induce mechanical cell lysis of adherent breast cancer cells (MCF-7).\(^{143}\) A more recent study by Kim et al. has shown that under excessive deformation of the deflecting membrane, small morphological changes known as “bulges” occur on the cellular membrane as a result of local detachment of the cytoskeleton lipid bilayer. In addition, differences in the uniformity of bulge distribution on the cell's periphery can be used as a physical indicator to distinguish different cells.\(^{144}\) A similar technology can also be used for viscoelastic characterization of cells of a small population in suspension. Du et al. estimated global viscoelastic time constants of the HL-60 cell line and 3T3 fibroblasts by observing the recovery time upon the removal of the compression force.\(^{145}\) This design can also be extended to the mechanical characterization of other specimens, such as biofilms.\(^{146}\) Alternatively, hydrostatic pressure can apply a uniform compressive force without physical contact.\(^{147}\)

Micropost arrays have been used to measure the traction force generated by a cell at the adhesion sites.\(^{148}\) The force applied by the cell to the substrate was calculated down to the level of a single adhesion site, based on the visualization of displacements and locations of the focal adhesions.\(^{148}\) Tan et al. used microfabricated microposts to determine mechanical cell-substrate interactions. By varying the geometry of the microposts, cell adhesion was controlled, and the results revealed that the magnitude of the traction force generated by the cell was regulated by its morphology.\(^{149}\) Schoen et al. investigated the effect of elastic substrate warping on force measurements. A correction factor was analytically and experimentally determined, which allows for a more accurate comparison of absolute forces between different pillar geometries and designs.\(^{150}\) In order to separately study the cellular response to external forces applied to a cell, and the internal forces generated by the cell, magnetic microposts containing cobalt nanowires that can be actively manipulated were developed.\(^{151}\) Fu and co-workers devised a strategy involving an array of microposts integrated onto a stretchable elastomeric membrane, enabling simultaneous stretching and contractile response monitoring of vascular smooth muscle cells (see Fig. 5(b)).

Due to the micro-scale dimensions of microfluidic channels, microchannels are suitable for generating fluid shear forces to study the substrate adhesion strength of cells. Several studies have quantified the adhesion strength of mammalian fibroblasts.\(^{153-157}\) These microfluidic devices share the commonalities of using microchannels with a protein coating to alter cell adhesion, which is tested under shearing fluid flow. Channels with varying widths were also developed to quantify cell adhesion strength.\(^{155,156}\) Using a similar tapered design, Gutierrez et al. demonstrated that activated neutrophils had a greater adhesion strength than their non-activated counterparts,\(^{157}\) and Rupprecht et al. used a tapered channel to quantify the adhesion strength of human breasts cancer cells (see Fig. 5(c)).

**Outlooks**

For clinical applications, cell samples from pleural fluid, urine and blood are highly heterogeneous, and the number of cells of interest is usually low (e.g., CTC cells). Thus, low-throughput techniques that are only able to test tens or hundreds of cells have little clinical relevance. In order to obtain statistically meaningful data, testing a large number of cells is necessary. Several techniques are able to provide reasonable throughput. For example, the constriction-channel device reported in ref. 39 achieved 100–150 cells s\(^{-1}\) and is capable of performing multi-parameter measurements. Since the measurements are completely electrical, by refining experimental parameters, a further increase in throughput could be feasibly achievable. The hydrodynamic stretching microfluidic device reported in ref. 19 has proven its clinical relevance and can provide a testing speed of approximately 2000 cells s\(^{-1}\). Although high-speed image recording and image analysis are a bottleneck, rapid advances in the imaging industry and hardware-based image processing could further enhance the clinical relevance of the microfluidic deformability cytometry technique.\(^{159}\)

Besides throughput, one challenge for microfluidic cell deformability measurements presently lies in the weak correlation between cell deformability and the widely used biochemical markers. An intrinsic connection between pathological cell deformability changes and their biochemical/molecular changes must be well established before cell deformability can practically be accepted as a clinical marker.

Cell deformability changes in response to environmental factors also needs to be better understood. After cells are harvested from the incubating environment or retrieved from the human body, the deformability of the cells starts to change. Many environmental parameters, such as temperature, \(\text{CO}_2\) concentration, and osmotic pressure, can have an influence on the deformability change. Thus, for cell deformability testing, testing conditions should be maintained as consistently as possible, and all cells should be tested within a strictly defined period of time. Establishing a standard testing protocol is necessary as microfluidic cell deformability testing moves towards clinical applications.

As for electrical measurements, microfluidic impedance flow cytometry appears promising since the conventional Coulter counter is a well-accepted technology. The DC/RF...
measurement technique has proven effective for clinical WBC differential count and is used in some commercial hematology analyzers. Thus, clinical acceptance and commercial success of microfluidic impedance flow cytometry could be achievable. As a precursor, instruments based on a miniaturized Coulter counter have entered the market. For instance, Chempaq (UNITECH, Denmark) is a portable hematology analyzer capable of 3-part WBC differential count, RBC count, and hemoglobin concentration measurements. Finger prick blood is loaded onto a disposable cassette, in which an aperture is placed. Only DC measurement is conducted by the instrument, and a 3-part WBC differential count is based on the modification of cell volume from chemical treatment.

Microfluidic impedance flow cytometry has proven more powerful than the microfluidic Coulter counter. Microfluidic impedance flow cytometry has been demonstrated to distinguish cells based on their physiological and pathological conditions, such as viability and membrane potential change.\(^\text{130}\) Although it is known that electrical properties of cells can possibly be used for distinguishing cell types (e.g., benign and malignant tumor cells), the establishment of the connections between cell physiological changes and their electrical properties has not gained as much interest as cell deformability research, likely due to the lack of powerful tools for cellular electrical characterization. Among the techniques discussed earlier in this review, only electrorotation is capable of quantifying a cell’s intrinsic (size and shape-independent) electrical properties (e.g., specific membrane capacitance and cytoplasm conductivity). However, electrorotation’s testing throughput is too low to generate meaningful results for diagnostic applications. Microfluidic impedance flow cytometry\(^{127,130–132}\) that uses opacity (ratio of high-frequency amplitude/low-frequency amplitude) as the indicator of a cell’s electrical properties can offer a high throughput. Opacity is a combined effect of the cell membrane and cytoplasm. A single RF frequency must be selected carefully so that different electrical properties of the cell membrane and/or cytoplasm can be well reflected in the opacity value. It is difficult to correlate electrical properties to physiological changes of cells since the opacity value can only reflect the electrical property changes of the cells. It cannot tell you what has caused the difference in opacity values and how the electrical properties have changed. In order to tackle this challenge, we recently reported a microfluidic device, which is capable of quantifying inherent electrical properties (specific membrane capacitance and cytoplasm conductivity) of single cells at a reasonable testing speed of 5–10 cells s\(^{-1}\) (vs. minutes per cell using existing techniques, such as electrorotation).\(^{139}\) Impedance profiles at seven frequencies were measured simultaneously when cells were aspirated through a constriction channel. Based on the geometrical and electrical models, specific membrane capacitance and cytoplasm conductivity values of over 6000 HL-60 and AML-2 cells were quantified.

In summary, we highlighted the recent developments of microfluidics-based techniques for the characterization of biophysical properties of single cells. Compared with conventional single-cell biophysical characterization tools, microfluidic devices have demonstrated great potential in realizing multi-parameter measurements on single cells at a higher testing speed. Biophysical measurements as a label-free approach can potentially be used for disease prescreening. For example, if biophysical measurements on cells indicate possible diseases, further clinical examinations can be done to evaluate the disease condition. Biophysical measurements can also be incorporated with a cell sorting unit to collect cells having different physical properties for further biochemical assaying.

While the throughput, sensitivity, and specificity of microfluidic biophysical characterization devices still await improvement, biophysical measurements on single cells already prove useful to provide bolstering information to biochemical measurements. Several microfluidic systems combining biophysical and biochemical measurements have been developed to capture multi-parameter information of the target cells.\(^{23,160,161}\) In the foreseeable future, closer correlations between biophysical and biochemical properties of cells will be established, and advances in microfluidics-based biophysical measurement techniques will move closer to practical applications and enable more discoveries in biology research. Meanwhile, as microfluidic devices for single-cell biophysical characterization are more integrated, compact, and easy-to-use, they can potentially become clinically accepted point-of-care tools.

**Acknowledgements**

Financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC) through an NSERC Steacie Fellowship, from the University of Toronto through a Connaught Innovation Award, and from the Canada Research Chairs Program is acknowledged.

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