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Acoustofluidic methods in cell analysis

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Abstract

Cellular analysis is a central concept for both biology and medicine. Over the past two decades, acoustofluidic technologies, which marry acoustic waves with microfluidics, have significantly contributed to the development of innovative approaches for cellular analysis. Acoustofluidic technologies enable precise manipulations of cells and the fluids that confine them, and these capabilities have been utilized in many cell analysis applications. In this review article, we examine various applications where acoustofluidic methods have been implemented, including cell imaging, cell mechanotyping, circulating tumor cell phenotyping, sample preparation in clinics, and investigation of cell-cell interactions and cell-environment responses. We also provide our perspectives on the technological advantages, limitations, and potential future directions for this innovative field of methods.

Keywords

Acoustofluidics; Microfluidics; Surface acoustic waves (SAW); Bulk acoustic waves (BAW); Cell analysis

1. Introduction

Cell analysis is an overarching process that describes the goal of assessing specific properties and/or dynamics of cells, either in isolation or as part of the tissue in multicellular or unicellular organisms [1]. Ever since cells were identified as the basic structural, functional, and biological unit of all known living organisms in 1800s [2], their analysis has served as the central concept for fundamental biology research and provided the basis for clinical diagnosis and therapeutics. As such, researchers have regarded the improvement of cell analysis techniques as a critical pathway to the advancement of biology and medicine. To this end, technical innovations have provided a consistent source of improvement for cell analysis techniques. Historically, the invention of the modern microscope led to the

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discovery of cells by Robert Hooke in 1665 [3]. This was the first instance of cell imaging, and this feat directly accelerated the establishment of cell theory [2]. Fast forward several hundred years, where advances in flow cytometry and fluorescence activated cell sorting (FACS) [4,5] in 1950–1960s allowed researchers to investigate and analyze the heterogeneity within cell clusters based on individual surface biomarkers. In the 1980s, the development of the polymerase chain reaction (PCR) technique by Kary Mullis *et al* enabled precision diagnostics based on the sequencing of unknown etiologies in cells [6]. As a more recent example, the maturation of droplet microfluidic technology has contributed to the achievement of single-cell RNA sequencing [7,8], which has been used to measure critical differences between individual cells at the transcription level within a cell population. It is clear that the future of precision cell analysis will depend upon technological innovations; it is just unclear of which area will provide the catalyst for this advancement.

Acoustic-based methods for manipulating particles and fluids in a microfluidic environment, termed acoustofluidics [9,10], have significantly advanced the field of cell analysis just as the numerous techniques that came before have done. In the 19th century, microparticles were used to visualize the distribution of acoustic waves, known as Kundt's tube [11,12], and ever since, researchers have relentlessly sought to harness acoustic waves as a tool to manipulate cells and extracellular fluidic environments. Acoustofluidic devices have become critical elements in the construction of cell analysis platforms; combining acoustofluidic manipulation techniques with cell characterization methods (*e.g.* PCR or imaging) enables precise manipulation and analysis in the microfluidic environment. In the last ten years, acoustofluidic techniques have been demonstrated for the patterning [13–17], focusing [18–21], separation [22–25], accumulation [26,27], sorting [28], and rotation of cells [29,30], and they have also been successfully applied in cellular analysis applications such as cell imaging [31], cell mechanotyping [32], circulating tumor cell (CTC) phenotyping [33], flow cytometry [34,35], and investigations into the interactions of cell-cell [36] and cell-environment [37,38] systems. It should also be mentioned that the development of acoustofluidic manipulation technologies is supported by (and not independent from) acoustic sensing, where changes in the frequency, amplitude, and phase of acoustic signals are used to analyze biochemical molecules and cells [39–41].

Acoustofluidic methods feature unique advantages for cell analysis applications, including operation with gentle force that will not disrupt normal cell physiology, and the ability to operate in a continuous manner with adjustable throughputs for both fundamental research and clinical applications on specimen ranging from bacteria to cells, and even multicellular organisms (ranging in size from nanometers to millimeters) in media including aqueous solutions, blood, and sputum. Acoustofluidic platforms also avoid the use of special working media, like those required in optical tweezers [42–44] or dielectrophoretic manipulation methods [45,46], which might damage cells. With these technical advantages in mind, we believe that acoustofluidic methods have the potential to provide many powerful tools and functionalities required for the next generation of cell analysis.

This review article aims to survey recent advances of acoustofluidic methods for cell analysis. Unlike other review articles in the field of acoustofluidics [10,47,56–60,48–55], this article focuses on acoustofluidic methods for cell analysis. Our discussion is categorized

by the cell analysis applications achieved (*e.g.* imaging or mechanotyping) rather than the acoustic operation (*e.g.* rotation or deformation) demonstrated. The scope of this work covers both surface acoustic waves (SAW) and bulk acoustic waves (BAW) as they share similar working mechanisms. Particularly, we will focus on whole, live cell analysis, instead of acoustofluidic cell lysis [61–63] or sonoporation [64–71]; we will focus on acoustic actuation rather than acoustic sensing [72,73]. To keep the discussion concise, acoustofluidic cell sorting are excluded, as they were discussed in earlier review articles [60]. A brief perspective and outlook on the future implications of acoustofluidic technology is also provided at the end of the article. We begin our discussion with applications of acoustofluidics in cell imaging.

2. Cell imaging

Microscopic cell imaging is one of the most inexpensive and effective methods to understand biological systems; it also set the fundamentals for many down-streaming technologies, such as cytometers. Simple bright-field analysis at the microscopic level can discern characteristics within cells and microorganisms that are sufficient for many types of analyses. Fluorescent capabilities greatly expand the applications and analysis that can be completed, where selective staining of biological components effectively enhances the specificity and intensity of the signal in observation. Furthermore, confocal microscopy provides benefits such as ultra-high spatial resolution in addition to capturing 3D images, which is crucial to the study of cell structures (such as the cytoskeleton); 2D slices of transparent samples also provide both internal and surface characteristics of the cell/model animals [74,75]. While modern microscopic methods may be powerful, they often suffer from two inherent limitations: (1) the throughput of observation is limited; and (2) the equipment for 3D imaging is expensive and bulky, prohibitive to many research groups. To overcome these limitations, acoustofluidic methods with rotational 3D imaging and parallel imaging have been developed.

The precise manipulation capabilities of acoustofluidic systems made them excellent candidates for rotational 3D imaging, where the body of interest rotates with acoustic streaming; capturing planar images throughout rotation can uncover features that may have been hidden from a single, static view. Ahmed *et al* leveraged the acoustic streaming created by an oscillating bubble that has been excited by BAW to rotate cells and model organisms [31]. In this work, the authors were able to achieve rotation of cells about a variable axis dependent upon bubble size and excitation frequency. Additionally, they achieved precise rotation of a model organism, *Caenorhabditis elegans* (*C. elegans*), with a rotational step size of 4° with a 5 ms pulse of the transducer. This rotation enabled the visualization of individual dendrites along the length of the worm using a fluorescent microscope. Ozcelik *et al* also leveraged BAWs to interact with the fluid domain [76]. In their device, the oscillations of solid sharp-edge structures, as opposed to trapped bubbles, were used to create acoustic streaming that rotated cells and organism. They also rotated *C. elegans* to achieve a 3D visualization using a 2D microscope. Schwarz *et al* [77] used three separated piezoelectric transducers to modulate the frequency, phase, amplitude, and directions of BAWs and rotated particle clusters parallel in microfluidic chamber (Fig. 1 A–B). Recently, Zhang *et al* used SAWs to achieve rotation of *C. elegans* (Fig. 1C) [29]. They achieved a 4°

rotation angle with a signal pulse of 1.5 ms, and even demonstrated rotation in a continuous flow (Fig. 1D). Trapping and rotation of cells and micro-objects using ultrahigh-frequency signals [30] or by leveraging acoustic potential fields [29] have also been reported.

Acoustofluidic technologies have also been utilized in high-throughput cell imaging platforms where acoustic forces are used to pre-focus the cells or conduct parallel cell manipulations. Zmijan *et al* [78] developed a BAW based method for acoustically focusing particles into a single flat layer, which mitigated issues associated with image blur due to a shallow imaging field of view. The device was capable of imaging in rapid flows with linear speeds up to 104 mm/s, resulting in a throughput of 208,000 beads per second. They also demonstrated the imaging of ATDC5 cells at ~60,000 cell per second and proposed their technology as a tool for CTC characterization. Another technique, known as "Imaging FlowCytobot (IFCB)," [79,80] utilized BAWs to focus cells into a line before passing the camera for imaging. This work successfully captured high-resolution (1 m) images and measured chlorophyll fluorescence of nano- and micro-plankton sized particles.

Thus far, rotational 3D imaging and high-throughput imaging enabled by the acoustofluidics has successfully expanded the capability of conventional microscopic imaging techniques. In order to take full advantage of these technologies, it is critical that researchers leverage the compatible nature of acoustofluidic technology; that is, these acoustofluidic tools need to be combined with additional microfluidic or micro-imaging technologies that will help to unlock their full potential. For example, integrating these acoustofluidic rotation platforms with any of the low-cost, or portable imaging platforms (such as cell phones) that have been developed recently [81] would remove the need for a standard benchtop microscope, making these tools more accessible in resource-limited environments. Additionally, combining acoustofluidic rotation with real-time image and analysis could allow researchers to screen specific cells/organisms which show desirable characteristics, and separate them for further analysis. For example, acoustofluidic rotation-based 3D imaging may be used to identify a *C. elegans* with a positive drug response. After identification, this worm could be isolated for downstream genetic analysis to identify markers that would justify the varied drug response. In summary, while the acoustofluidic-based 3D imaging tools are powerful, they can be taken further and integrated with other technology to improve their relevance.

3. Cell mechanotyping

The characterization of cell mechanical properties (*i.e.* cell mechanotyping) is valuable for both diagnostic and cell biology research efforts. Changes in cells' mechanical properties are usually associated with multiple diseases such as cancer, malaria, arthritis, atherosclerosis, hypertension, cerebral edema, stroke, and asthma [82–86]. Several biomechanical characteristics, including deformability, Young's modulus, and compressibility are used to represent cell mechanical properties. These mechanical properties are conventionally measured using either an atomic force microscope (AFM) or optical tweezers (OT). When using an AFM, cells are linked with the tip of AFM probes and stretched precisely; using OTs, cells are conjugated with microparticles and deformed using optical radiation forces. Although these methods provide exceptional resolution in displacement and force measurement, AFM and OT methods suffer from low throughputs (~1 minute for a single

cell). Therefore, while these methods may be sufficient for some fundamental biological studies, they are less applicable to many other applications such as clinical diagnoses. Acoustofluidic devices provide an alternative approach to AFMs or OTs for cell mechanotyping.

Cell deformability can be measured by stretching cells in acoustic field. As an example, Xie *et al* (Fig. 2A–B) developed a method that utilizes an acoustically oscillating bubble to deform cells suspended in an acoustic streaming field and assess their deformability [32]. This method measured mechanical biomarkers from multiple cells in a single experiment. Specifically, using this technique, the mean deformability of tens of HeLa, HEK, and HUVEC cells were assessed to distinguish their mechanical features. The method was easily integrated with other bioanalysis and drug-screening platforms; for example, the mechanical response of Hela cells after treatment with Cytochalasin was demonstrated. This acoustofluidic technique was also able to uncover the deformability of each subpopulation in a mixed, heterogeneous cell sample by using both fluorescent markers and mechanical biomarkers. Furthermore, a recent work demonstrated that the stiffness (*i.e.* elastic modulus) of single suspended cells can be measured by the shift of resonance frequency of a suspended microchannel resonator; this method is dependent upon acoustic scattering [87].

Acoustofluidics also provides a unique mechanism to measure cell compressibility by using the acoustic radiation force. Theoretically, a particle/cell exposed to an acoustic field experiences an acoustic radiation force given by [33]:

$$F_a = -\left(\frac{\pi p_0^2 V_p \beta_m}{2\lambda}\right)\varphi(\beta,\rho)\sin(2ky)$$

$$\varphi(\beta,\rho) = \frac{5\rho_p - 2\rho_m}{2\rho_p + \rho_m} - \frac{\beta_p}{\beta_m}$$

where p_0 and V_p are the acoustic pressure and the volume of the particle, respectively; λ and k are the wavelength and the wave number of the acoustic waves, respectively; and φ is the acoustic contrast factor, which is dependent on the compressibility and density of the particle (β_p and ρ_p, respectively) and the medium (β_m and ρ_p , respectively). In a typical mechanotyping experiment, the medium properties, excitation frequency, and pressure amplitude are known; V_p is assessed with microscopic observation; F_a is calculated using cell trajectories in the acoustic field; ρ_p is estimated from literature. Knowing all of the other factors, the cell compressibility (β_p) can be readily calculated. For example, Hartono *et al* [88] recorded cell trajectories in a BAW field and obtained the compressibility of various cell types. They found that the compressibility of cancer cells is higher than that of normal breast cells, indicating that compressibility could be used as a diagnostic marker for cancer. To simplify the experiments, Wang *et al* [89] infused cells from the same initial positions and recorded a deflection-cell size relationship in a BAW field that indicated cell compressibility. They revealed differences in multiple cell lines with a throughput of 10 cells per second (Fig. 2C–D). In another work, iodixanol was introduced to alter the acoustic

properties of the cell-culture medium such that cells can have positive, zero, or negative acoustic contrast depending on the molecular concentration. Manipulated by the BAW field, cells migrate to their iso-acoustic point, where their positions are indicative of their compressibility (Fig. 2E) [90]. In addition to cells, acoustofluidic mechanotyping was also applied to larger animals. Baasch *et al* [91] measured the compressibility of *C. elegans* in a BAW field, and correlated it with the volume fraction of lipids and proteins that would mainly make up the body of *C. elegans*. The above-mentioned cases exemplify that acoustofluidic methods have unique advantages in cell mechanotyping. They are contactless and can more readily achieve higher throughputs compared with traditional techniques such as OT and AFM. Acoustofluidic methods can also measure cell compressibility and the bulk modulus (*i.e.* inverse of compressibility) using the acoustic radiation force, in addition to the conventional deformability measurements.

In addition to measuring deformability and compressibility of individual cells, the strength of cell adhesion can be characterized with acoustofluidic methods as well. In a technique named “Acoustic Force Spectroscopy”, cells that have been bound to a microfluidic surface are levitated using a BAW toward the position of a pressure node. The displacement of cells and input power required to achieve the displacement were correlated and used to calculate adhesion strength. The authors demonstrated exertion of controlled forces, up to 1 nN, onto hundreds of individual cells in a parallel fashion [92]. It was determined that CD4 adhesion is accelerated by interleukin-7, remaining CD4 binding strength the same [92]. “Acoustic Force Spectroscopy” was also used to uncover protein-DNA interactions [93,94], and red blood cell deformability [95]. In these cases, the displacements of cells/DNAs that were linked to a microparticle were recorded when exposed to an external BAW field.

Despite its advantages in versatility for cell mechanotyping, the choice of which biophysical parameter to measure, and the conditions under which the measurement is should be carefully considered. First, one must consider whether the compressibility or deformability is a more accurate biomechanical marker for their application. Most reports indicate that cancer cells are both more compressible and more deformable compared with benign cells [85]; from this information, it would appear that the cell compressibility and deformability are positively correlated. However, Ravetto *et al* [96] revealed that monocytic cells become less compressible but more deformable upon exposure to inflammatory chemokines, confounding the simple relationship that researchers might have taken for granted. Researchers must also consider whether measurements should be taken *in situ* or when cells are suspended in a microfluidic channel. Unlike AFM or OT, current acoustofluidic mechanotyping procedures are not conducive to *in situ* experimentation; this means that cell handling and medium exchanges might alter the mechanical properties of cells before testing. These are just two of the factors that need to be considered when choosing to implement an acoustofluidic strategy in a cell mechanotyping experiment; nonetheless, acoustofluidics provides an excellent, biocompatible, high-throughput method for cell mechanotyping experiments.

4. Circulating tumor cell phenotyping

CTCs are an extremely rare type of cancerous cells which escape from a primary tumor and are carried around the body with blood circulation [97–100]. The CTCs induce metastasis, a major cause of cancer-related death, by acting as a seed for subsequent growth of tumors on distant organs. Despite the importance of analyzing CTCs for cancer prognosis and research, one major barrier for CTC phenotyping is their extremely rare nature in blood. Researchers have estimated the number of CTCs in the blood of patients with metastatic diseases to be on the order of 0–100 CTCs per mL. Therefore, developing methods to isolate CTCs from the vast number of other blood cells is imperative for successful CTC analysis. Cellsearch, a macro-scale immunomagnetic-based approach, is currently the first and only method approved by the U.S. Food and Drug Administration to isolate CTCs. Aiming to circumvent the limitation surrounding the use of surface biochemical markers (*e.g.* EpCAM) and obtain live CTCs, acoustofluidic methods have been developed to isolate, enrich, and phenotype CTCs based on differences in size and/or compressibility between CTCs and other cell types in blood.

In the last five years, acoustofluidics have been proven as one of the most promising methods for CTC phenotyping. Li *et al* [33] developed a SAW-based method that was capable of separating CTCs in peripheral blood samples obtained from cancer patients. They used a tilted-angle standing SAW field to deflect cells to different outlets based on their size difference. This method can separate low concentrations (~100 cells/mL blood) of a variety of cultured cancer cell lines from white blood cells (WBCs) with a recovery rate better than 83% and a flow rate of 1.2 mL/h. The isolation of CTCs in blood samples obtained from patients with breast cancer was demonstrated as well (Fig. 3A–C). Recent work from the same group improved the throughput of SAW-based CTC separation [101]; the revised design employed a poly-dimethylsiloxane (PDMS)-glass hybrid channel, as an acoustic resonator, to increase the energy density and the resulting throughput. It also featured a divider in the channel to decrease the velocity of cells, thereby increasing the travel time of the cells within the SAW field and improving CTC separation efficiency. The separation throughput was increased to 7.5 mL/h, comparable to current clinical CTC assays (*i.e.* Cellsearch). In addition to SAW-based methods, BAW techniques have also been exploited extensively to separate cancer cells from blood cells [102–105]. For example, with a BAW-assisted cell focusing, separation, and enrichment protocol, Antfolk *et al* [102] demonstrated that breast cancer cells (MCF7 cells) spiked into red blood cell-lysed human blood were separated with an efficiency of 91.8 ± 1.0% at a flow rate of 100 L/min. The recovery rate of prostate cancer cells (DU145 cells) spiked into whole blood was 84.1 ± 2.1% (Fig. 3 D–E); the same group improved their device design by eliminating the need for a secondary medium to hydrodynamically pre-position cells before the separation, and reached an 86.5 ± 6.7% recovery rate for the cancer cells (DU145) spiked into blood [103].

There is great promise, as well as many challenges faced when using acoustofluidics for CTC phenotyping. BAW and SAW based devices both provide contactless, biocompatible, and compact methods for isolating CTCs from blood samples [100]. However, the CTC separation performance is depended upon the size and compressibility of the individual cells. This might not be a technical limitation, but it is necessary to define what type of

CTCs are suitable for acoustofluidic separation/analysis, which requires extensive background knowledge of specific cancers and their CTCs. Additionally, current acoustofluidic methods are expected to integrate with downstream molecular characterization of CTCs. Mere enumeration, as current results of CTCs phenotyping, has had yet to enhance clinical prognostication or diagnostics in a meaningful way; these devices should be explored in conjunction with molecular analyses like transcriptomic or proteomic analysis [106] to truly impact cancer diagnosis, prognosis, and treatment.

5. Cell interaction

Cells form organized tissues and organs through precisely regulated interactions including cell-cell and cell-environment associations. Understanding physiological and pathological cell interactions is of great importance for diagnosis and therapeutics of diseases such as cancer and diabetes [107,108]. Uncovering this information requires techniques that are capable of manipulating cells and/or the surrounding fluidic environment with high resolution both spatially and temporally [109,110]. Recently, researchers have made great efforts in developing acoustofluidic devices to probe cell-cell interactions and cell-environment responses [56].

With regards to cell-cell interactions, Li *et al* [111] developed a SAW-based cell co-culture platform to alternatively seed and culture two cell lines; in this platform, one type of cells was patterned on the chamber surface using SAWs; then, a phase-shift of the SAW field was implemented to move the pattern of the second cell type in between the node-lines of the first cell type. Co-culturing HeLa and HMVEC-d cells led to the discovery of increased cancer cell mobility, which might be attributed to the cross-talk initiated by endothelial cells which enhances cancer cells through STAT3/Akt/ERK, α5β1 integrin, and GTPases signaling pathways (Fig. 4A–B). Kang *et al* [112] used a SAW-based method to fabricate therapeutic vascular tissue containing a three-dimensional collateral distribution of vessels. Co-aligned human umbilical vein endothelial cells and human adipose stem cells were arranged in a biodegradable catecholconjugated hyaluronic acid hydrogel; this arrangement enhanced cell-cell contact, gene expression, and secretion of angiogenic and anti-inflammatory paracrine factors (Fig. 4C). SAW-based methods have also been used to investigate cell-cell interactions at the single cell level. Guo *et al* [36] demonstrated a SAW-based approach to control the distance and spatial arrangements of a few suspended cells, and quantitatively investigated the gap junctional intercellular communication in several homotypic and heterotypic populations by visualizing the transfer of fluorescent dye between cells (Fig. 4D–E). The SAW-based method was also applied towards understanding how individual human lymphocytes and red blood cells are effected by the malarial parasite *Plasmodium falciparum* (Fig. 4F) [113]. Recently, investigation of cell interactions in more complex, 3D spheroids, was demonstrated with SAWs in order to investigate tumor growth and treatment [114–116]. Chen *et al* [114] discovered that the existence of HepG2 cells on the outer surface of spheroids protected the inner cells from the anti-cancer drug 5-fluorouracil. The work on spheroids mimics cancerous tissues in humans and elucidates that tumor spheroids feature distinct drug resistance due to their 3D structure (Fig. 4G).

With regards to cell-environment responses, progress has been made toward the spatial modulation of biochemical and/or biophysical stimuli with BAWs and SAWs through acoustic streaming-based fluid manipulation. For example, Ahmed *et al* [38] demonstrated an oscillating bubble-based, BAW approach to generate programmable chemical waveforms that permitted continuous modulation of the signal characteristics including the amplitude (*i.e.* concentration of chemical stimuli), shape, frequency, and duty cycle, with frequencies reaching up to 30 Hz. Using this method, they demonstrated a frequency-dependent activation and internalization of G-protein coupled receptor internalization on HEK 293 cells. To overcome the instability of the oscillating bubble, Huang *et al* [37] employed a sharp-edge-based BAW method to generate chemical signals. Chemical signals were precisely controlled with periods from 100 ms up to several hours. This method was applied later in cell signaling studies by probing the dynamics of calcium release stimulated by ionomycin signals in HMVEC-d, HeLa, and U-251 cells. They found that a short single-pulse ionomycin (100 ms) allowed cells to dynamically adjust the intracellular level of Ca^{2+} through constantly releasing and accepting Ca^{2+} to the cytoplasm and from the extracellular environment, respectively (Fig. 5A–C). In addition to BAW methods, SAW-induced acoustic streaming has also been used to quantify the interaction of protein-coated particles with cells, in order to evaluate performance of drug delivery vehicles under physiological flow conditions (Fig. 5D–F) [117]. In terms of cellular responses to biophysical stimuli, Zhang *et al* showed F-actin cytoskeletal rearrangement in MC3T3-E1 cells and an increase in intracellular calcium concentration with application of an acoustic radiation force generated by SAWs [118]. Greco *et al* [119] reported that U-937 monocyte cell proliferation under acoustic streaming from SAWs was enhanced by 36% with respect to those of standard static cultures. Stamp *et al* [120] demonstrated that SAW-treated, osteoblast-like SaOs-2 cells exhibit a significantly increased migration, promoted cell growth, and stimulated wound healing, as compared to the control samples. The above-mentioned examples clearly demonstrated the contributions from acoustofluidic devices in understanding cellular response to extracellular biochemical/biophysical signals [121–123], which have great potential in developing future therapeutics [124–129].

6. Streamlined cell analysis from clinical samples

Owing to the complex, potentially viscous nature of many biological samples (*e.g.* whole blood, stool, or sputum), many acoustofluidic devices require external equipment to process the sample before it can be successfully injected into the microfluidic domain and function properly. This dependence on benchtop equipment negates many of the benefits, including continuous operation and biosafety, gained when using an all-in-one, integrated microfluidic platform. This has led researchers to develop methods that enable the handling of these complex samples.

The development, commercialization, and application of acoustic focusing to flow cytometry efforts exemplifies this process [130]. Although research started with simple acoustic focusing of particles in a cylindrical tube, commercial implementations have integrated hydrodynamic and acoustic focusing together with 4-laser optical detection and mechanical-electric systems to develop complete cytometry systems [34,131]. This has enabled high precision analysis of cells with volumetric sample input rates up to an order of magnitude

higher than systems that forgo acoustic-assisted focusing for hydrodynamic focusing alone [132,133].

As another example, recent publications from Huang and his colleagues demonstrated a series of acoustofluidic devices that can perform sputum liquefaction, cell transfer, and inflammatory cell analysis using human sputum samples. They first demonstrated a sharp-edge-based BAW device capable of liquefying sputum samples to facilitate further cell handling [134]. Their device combined dithiothreitol, a mucus liquifying reagent, with sputum samples using acoustic streaming that was induced by oscillating sharp-edge structures; the strong acoustic streaming produced by the device was able to liquefy sputum effectively at a throughput of 30 μL/min, while cell viability and integrity were well maintained (Fig. 6A). After liquefaction, the cells remain mixed with the potentially harmful dithiothreitol, which necessitates a centrifugation step to remove the cells. In order to avoid this step, and keep the process solely dependent on compact microfluidic tools, the same group created a SAW-based platform to transfer the cells from the liquefied sputum sample to culture media or a buffer solution [135] to avoid the toxicity of residual dithiothreitol, and prepare for cell cytometry and immunostaining analysis (Fig. 6B). These works demonstrated the capability of acoustofluidic devices in dealing with complex, highly viscous, and delicate biological samples. It also showed that acoustofluidic devices can prove advantageous when integrating up/down-stream cell handling and analysis platforms. The application of this method was later expanded to liquify and purify stool samples for bacterial identification [136]. The combination of multiple, functionalized acoustofluidic units greatly expands the capabilities for handling complex clinical samples, and provides numerous benefits compared to traditional, benchtop analysis. Nonetheless, current designs have only been shown to work as discontinuous sub-units, meaning that future innovation and integration is needed to achieve a completely cohesive system that realizes the full potential of acoustofluidic technologies in cell analysis.

7. Perspectives

Acoustofluidic methods feature several advantages for live cell analysis. Acoustofluidic methods are able to maintain excellent cell viability during manipulation. Both SAWs and BAWs work with low power intensity (*e.g.* ~0.1 W/cm^2) [10], similar to clinically applied ultrasound tests, inducing minimal heating or shear forces that might cause cell damage [63]. They work effectively with almost any biofluids such as cell culture medium [111], sputum [134], stool [136], blood [23,138], and milk [50], in contrast to the requirement of special medias in many traditional methods such as optical tweezers and dielectrophoresis; this feature help to maintain normal cell functions during cell analysis. In addition, acoustofluidic methods can manipulate both cells and extracellular environments. Acoustic radiation forces can be used to [48,139] deflect cells for separation, or trap cells for patterning, contacting, and forming spheroids [13]; on the other hand, acoustic streaming [49,140] can be used to deform cells for mechanotyping [32], rotate cells for imaging [29–31,76,141,142], or actuate fluids to manipulate extracellular environments [37,143]. Acoustic waves feature proper resolutions and a suitable wavelength size range for cell manipulations. For example, an acoustic wave with a 10–100 MHz frequency produces a wavelength of 15–150 micrometers in water. This allows researchers to manipulate whole

cells, and precisely move cells by distances of about 1–1000 micrometers, which is suitable for many applications such as cell separation, mechanotyping, and interaction. The biocompatibility, versatility, resolution, and simplicity make these acoustofluidic methods superior platforms capable of handling a wide range of applications in biology and medicine.

Despite the rapid growth and intriguing applications, there are a few considerations for future developments of acoustofluidic-based cell analysis methods in fundamental and clinical research efforts. First, the complexity of cells, diseases, and biological processes must be properly recognized. Similar diseases may yield distinct cell analysis results. Take CTC analysis as an example, there is no single characteristics that could be applied for all types of CTCs. In the Cellsearch method, the application of EpCAM positive selections is valid for the monitoring of patients with metastatic breast, colorectal, or prostate cancer, but has limited performance in detecting lung cancer [144,145]. Similarly, acoustofluidic methods separate cells based on size, compressibility, or other physical properties, where the best performance might only be achieved for certain types of cancer. Thus, it is essential to define the scope of applications and standardize the operation protocol accordingly. In addition, similar results of cell analysis might be caused by distinct biological mechanisms. Take cell mechanotyping as an example, changes in cell deformability and compressibility might be associated with altered compositions of plasma membrane, cytoskeleton structure, cell-phase, or parasite invasions [85,86,146]. In another case, cell-cell communication can be attributed from physical contact, diffusion of soluble factors, transmission of electrical signal, and transduction of mechanical cues within the extracellular matrix [147]. In both cases, it may be difficult to correctly assign the reason for the cellular responses; correct interpretation of the results requires integration of multiple analytical methods, proper design of control experiments, and development of approaches to separate targeted signals from the background noise. Here, both BAW and SAW devices are promising for use in combination with other molecular biology or analytical chemistry methods to enhance the accuracy of data interpretation. Furthermore, the complexity of cells must be considered when studies involve vast types of cell specimens. Current acoustofluidic methods deal with single cells, 2D/3D cell cultures, and small animals (*i.e. C. elegans*); however, more specimen types such as tissues, organoids, organ slices, and cells *in vivo* remain mostly unexplored. Additionally, most research efforts are carried out with cells merged in liquid; however, a large portion of cells function only at liquid-air interfaces (*e.g.* skin, eye, and lung) [148,149] which have yet to be investigated with acoustofluidic methods.

The idea of integrating multiple methods for accurate cell analysis brings about the second consideration: to develop acoustofluidic system for fundamental cell biology studies. In this regard, special emphasis should be paid to the logical and physical interfaces between acoustofluidic units and other analytical methods up- and down-stream. Since cells are usually analyzed with multiple approaches, the ability to integrate with other biochemical characterization methods is desirable. For example, in a typical cell biology study, cells that are exposed to external stimuli are often investigated by considering transcription (*via* RT-PCR), proteomics (*via* mass spectra), histology (*via* fixation and staining), and cell cultures. Other than real-time *in situ* observations, most procedures require collecting the whole or a selective portion of cells from the microfluidic chamber, which is not readily feasible for

traditional PDMS-based microfluidic devices. To overcome this limitation, recent acoustofluidic devices have been fabricated using disposable chips [150,151], glass capillaries [152], flexible substrates [153], as well as open droplets [14]. These devices share similar mechanisms and performance standards to early acoustofluidic platforms but successfully remove PDMS barriers. We expect that future cell analyses will be conducted on fully integrated platforms where the acoustofluidic fraction plays an essential, but not exclusive, role in the procedure.

Finally, to develop acoustofluidic systems capable of functioning outside of centralized labs, the device design, fabrication, and peripheral equipment must be modified to suit the application environment. Despite the remarkable research-laboratory performance, there are few commercially available acoustofluidic products for cell analysis [34]. Part of the reason for this disconnect might be that current acoustofluidic devices still heavily rely on expensive, complicated equipment such as function generators, amplifiers, and infusion pumps. Reducing the dependence of acoustofluidic devices on these pieces of equipment based on specific applications will broaden the range of settings where acoustofluidics can be relevant. For point-of-care diagnostics that occur at homes, nurse stations, or other resource-limited settings, microscope-free, pump-free, and function-generator-free systems are desirable. Although challenging, efforts have been taking to simplify the supportive equipment. For instance, Huang *et al* [154] invented a programmable acoustofluidic pump, to replace conventional syringe pumps, that utilized the acoustic streaming effects generated by the oscillation of tilted sharp-edge structures. Later, with the use of a cell phone, a modified Bluetooth® speaker, a sharp-edge-based acoustofluidic device, and a simple portable microscope, Bachman *et al* [155] developed an on-demand acoustofluidic pump and mixer, and prototyped with commercial available Arduino platforms [156] that was promising for point-of-care applications. With efforts from academia and industries, we expect that a broader application of acoustofluidic methods will boost both fundamental and clinical cell studies.

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- Acoustofluidics provides benefits comparing to conventional cell analysis methods.
- Surface acoustic waves and bulk acoustic waves are two major acoustofluidic methods.
- Examining applications that utilize acoustofluidics to analyze whole, living cells.
- Discussing advantages, limitations, and outlooks for acoustofluidic cell analysis.

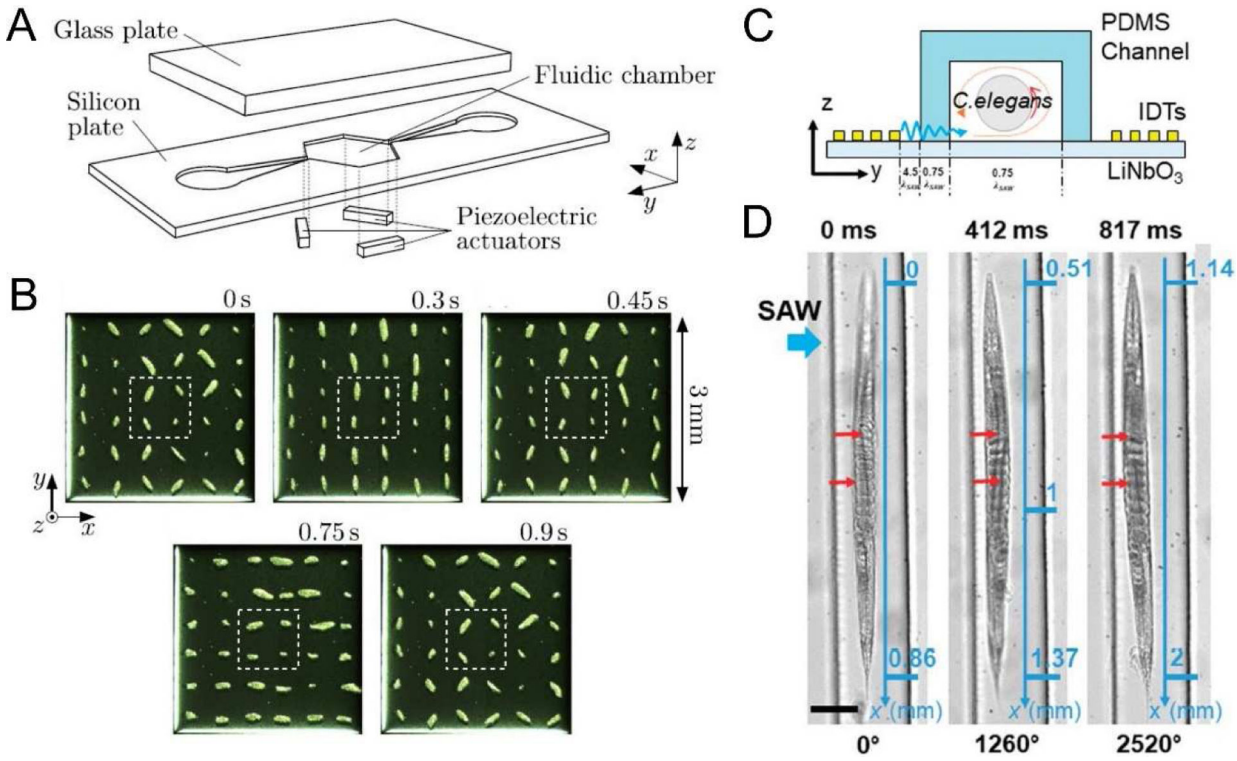

Figure 1.

(A) A BAW-based acoustofluidic device that rotates particles with acoustic radiation torques; (B) A 180 rotation of 36 particle clumps formed out of 17 μm particles achieved using a phase modulation. (C) A SAW-based device for *C. elegans* rotation; and (D) rotation of a *C. elegans* in a continuous flow *via* acoustofluidics. Images reproduced with permission from references [29,77].

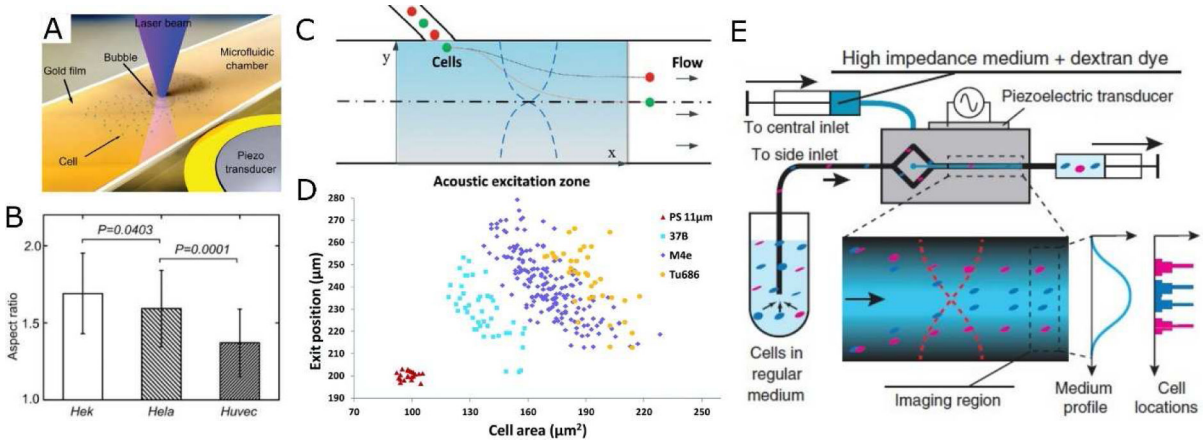

Figure 2.

(A-B) Probing cell deformability with acoustic streaming near an oscillating bubble. (C-D) Probing cell compressibility with deflection in a BAW-based device. (E) Probing cell impedance with acoustic contrast gradients in a BAW-based acoustofluidic device. Images reproduced with permission from references [32,89,90].

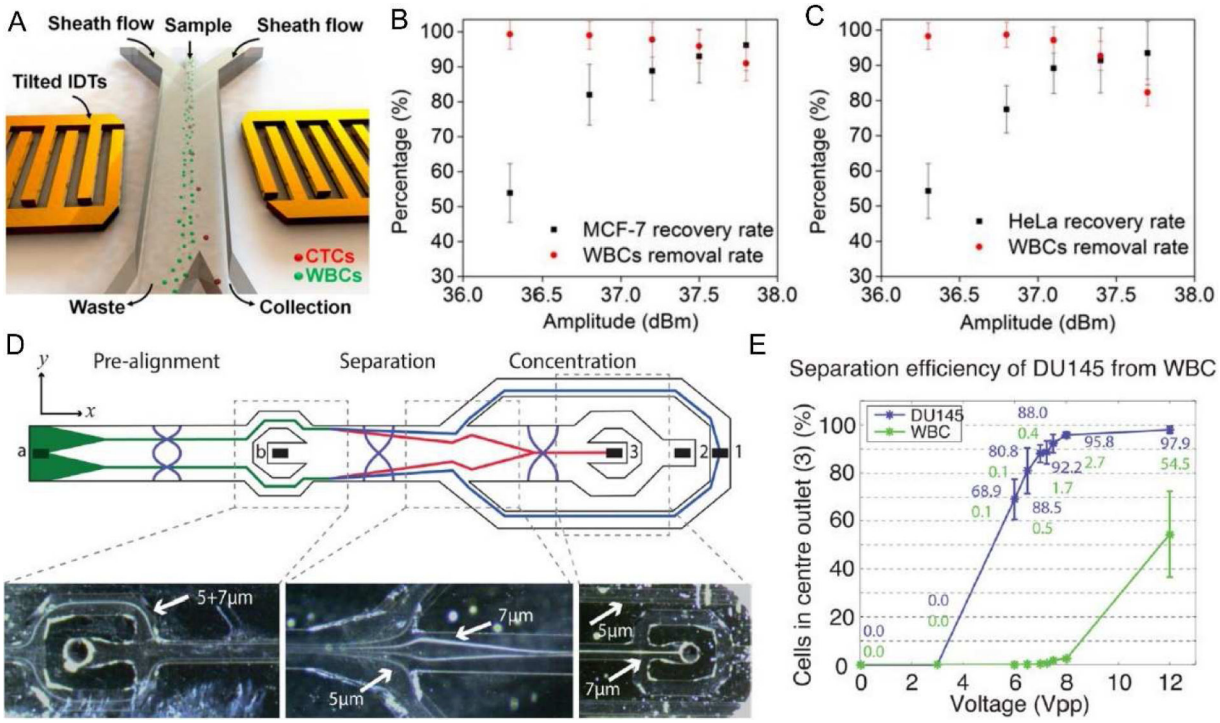

Figure 3.

(A) SAW-based CTC separation and phenotyping. Performance for separation of (B) MCF-7 cells from WBCs, and (C) HeLa cells from WBCs at different power inputs. (D) Schematics and trajectories of 5 and 7 μm polystyrene particles in a BAW-based CTC separation device. (E) Performance for separation of DU 145 cells from WBCs at different power inputs. Images reproduced with permission from references [33,102]

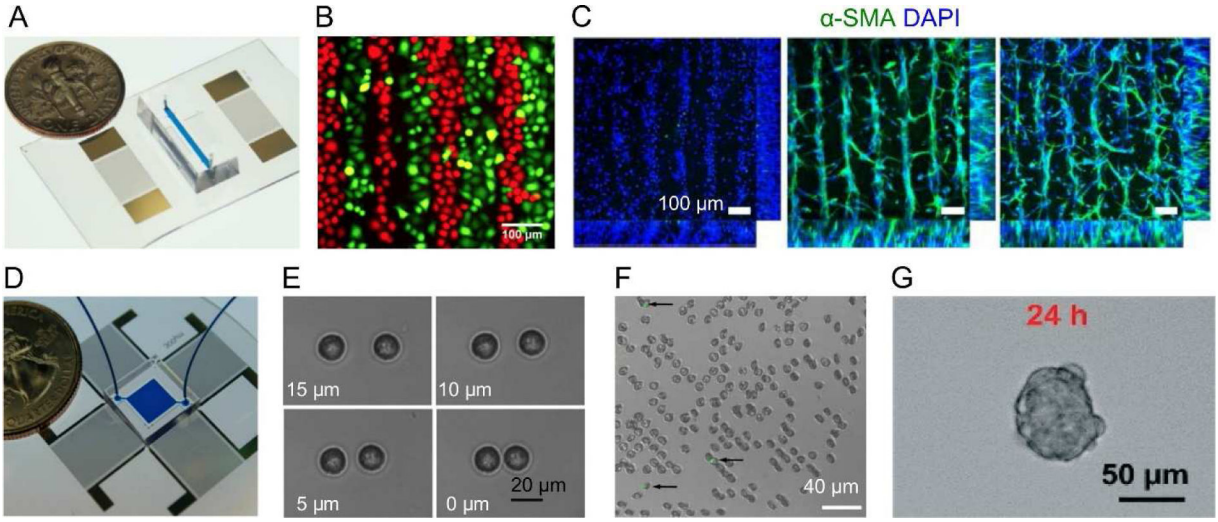

Figure 4.

Acoustofluidic methods to probe cell interactions. With (A) two parallel interdigital transducers, (B) two cell types can be co-cultured in adjacent lines, (C) co-culturing HUVEC/hADSC in ratios of 1:0, 5:1, and 2:1 demonstrated enhanced cell–cell contact in the fabricated cell-hydrogel construct for studying vessel maturation. (D) With four transducers in a square configuration, cells can be manipulated individually to investigate (E) cell-cell interactions, (F) cell-malarial parasite interactions, and (G) cells in a 3D spheroid. Images reproduced with permission from references [36,111–114].

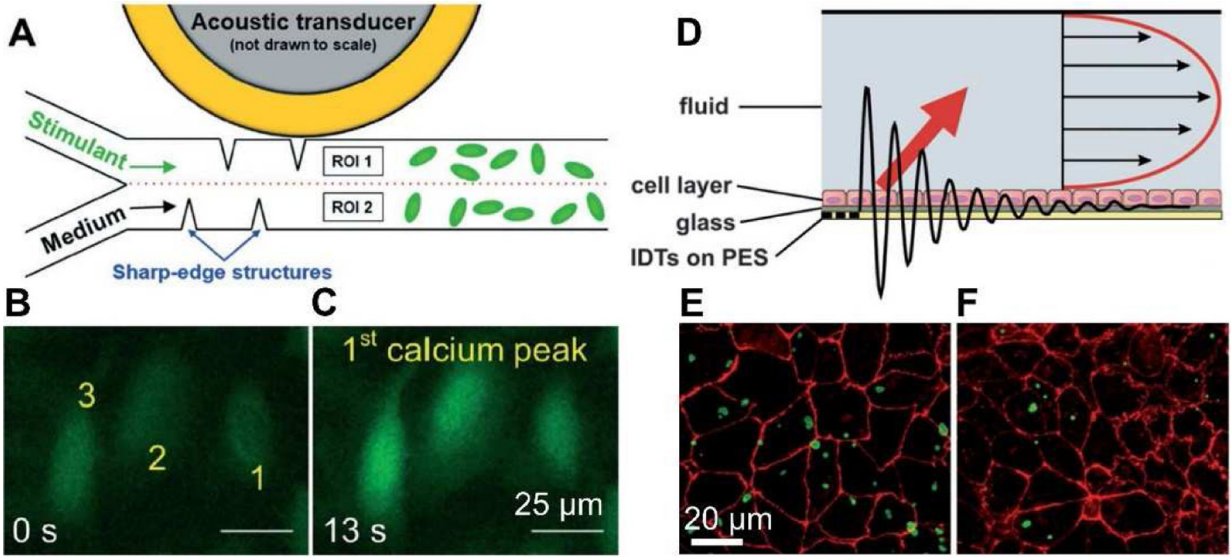

Figure 5.

Acoustofluidic methods to investigate cellular responses to the environment. (A) acoustic-actuated sharp-edge-based BAW methods to produce chemical waveforms by acoustic streaming. (B-C) Change in fluorescence intensity of three representative HMVEC-d cells with periodic ionomycin stimulation. (D) SAW-based method to generate acoustic streaming. (E-F) Detachment of wheat germ agglutinin coated microparticles from cell surfaces under stationary conditions and flow conditions. Images reproduced with permission from references [37,117].

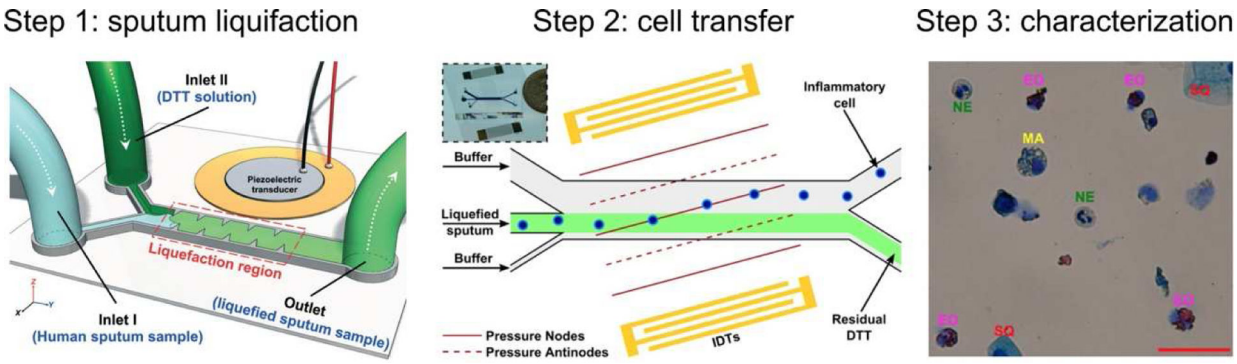

Figure 6.

Acoustofluidic devices are used to streamline the processing and analysis of human sputum samples. (A) In the 1st step, an acoustofluidic device is used to liquify sputum sample with acoustic streaming. (B) In the 2nd step, an acoustofluidic device is used to transfer cells from liquified sputum to cell culture medium for analysis. (C) A histology study was conducted to characterize cell types in the 3rd step. Images reproduced with permission from references [134,135]