Chapter

Preparation of Tissues and Heterogeneous Cellular Samples for Single-Cell Analysis

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Abstract

While sample preparation techniques for the chemical and biochemical analysis of tissues are fairly well advanced, the preparation of complex, heterogenous samples for single-cell analysis can be difficult and challenging. Nevertheless, there is growing interest in preparing complex cellular samples, particularly tissues, for analysis via single-cell resolution techniques such as single-cell sequencing or flow cytometry. Recent microfluidic tissue dissociation approaches have helped to expedite the preparation of single cells from tissues through the use of optimized, controlled mechanical forces. Cell sorting and selective cellular recovery from heterogenous samples have also gained traction in biosensors, microfluidic systems, and other diagnostic devices. Together, these recent developments in tissue disaggregation and targeted cellular retrieval have contributed to the development of increasingly streamlined sample preparation workflows for single-cell analysis technologies, which minimize equipment requirements, enable lower processing times and costs, and pave the way for high-throughput, automated technologies. In this chapter, we survey recent developments and emerging trends in this field.

Keywords: tissue dissociation, cell sorting, microfluidics, diagnostics, devices

1. Introduction

The common conception of *in vitro* diagnostics is intertwined with the idea of a liquid starting sample - blood, saliva, urine, and other starting materials are often the candidates for study, allowing rapid determination of important details about a patient's health status by investigating metabolomic, proteomic and genomic markers of disease [1]. Tissue samples are comparatively less discussed, with much less research devoted to optimizing their sample preparation for analysis. Despite the comparative lack of research in this area, tissue specimens are ubiquitous diagnostic samples, particularly for cancer, where they are used to confirm and characterize almost every case of solid-tumor cancer (Mayo Clinic, 2020).

While liquid biopsy has seen much recent innovation as a potential cancer diagnostic, the paucity of relevant tumor cells in the collected liquids often fails to adequately represent the cellular heterogeneity present within cancer tissues. Consequently, while liquid biopsy, diagnostic imaging and other tests are often conducted for preliminary diagnosis of cancer, tissue biopsies and cancer tissue specimens remain the standard and are routinely needed in order to profile an

individual's cancer and assess prognosis, metastasis, treatment options, and more. As such, millions of tissue biopsy procedures are conducted every year in the United States.

In the current practice, repeat tissue biopsies are often required and much of the biopsied materials end up being wasted. Sources have estimated that the United States spends $8 billion annually on unnecessary repeat biopsy procedures just for breast cancer [2]. This is often because bulk sequencing and histopathological analyses limit the full extent of investigation. There is a growing interest in applying Single-Cell Analysis (SCA) techniques to better understand tissue heterogeneity and to improve the efficiency of sample recovery in tissue diagnostics. SCA is an umbrella term for any tests that ascertain cellular characteristics at the individual cellular level. The most popular emerging SCA approach for cancer diagnostics is arguably Single-Cell Sequencing (SCS) of nucleic acids (DNA, RNA), which also encompasses techniques such as single-cellRNA sequencing (scRNAseq). This is a Next Generation Sequencing (NGS) approach to characterize the genomes or transcriptomes of individual cells.

While traditional approaches involve homogenizing whole tissue sections and extracting DNA from bulk tissues, SCS techniques and traditional genomic or transcriptomic approaches involve isolation of single cells for sequential high throughput analysis. Because of this, there is a growing interest in using SCS techniques for cancer diagnostics, as they are able to significantly reduce background noise and improve resolution [3]. However, a critical component which presently limits the clinical translational potential of the SCS workflow remains under-investigated: How does one get from a complex, heterogeneous section of tissue to a suspension of targeted cancer cells? We herein provide an overview of the different approaches whereby suspensions of single targeted cells can be prepared from complex tissues, with an emphasis on applications that target advanced SCS analysis (**Figure 1**).

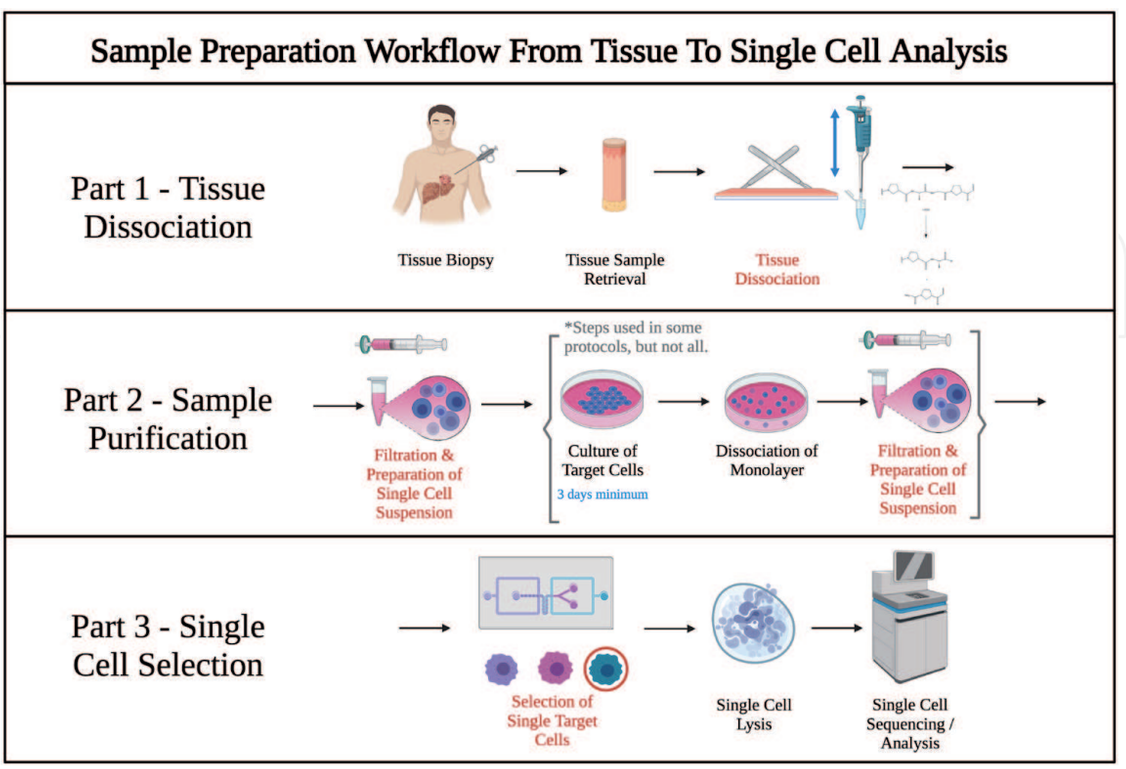

Figure 1.
Overview of the conventional sample preparation workflow from tissue to single cell sequencing. Topics in red will be discussed at length in this chapter.

2. Tissue dissociation methods

2.1 Introduction to conventional tissue dissociation

We will begin by summarizing the conventional approaches currently used for tissue dissociation - the process of obtaining cellular suspensions from tissues. We will discuss recent innovations at the academic and commercial level and will conclude by discussing some possible future directions for innovations in this area.

Current methods for dissociating tissues into single cells rely on chemical dissociation techniques, mechanical dissociation techniques, or a combination of the two. The simplest benchtop preparation methods often consist of mincing tissues repeatedly with scalpels, followed by chemical treatment with proteases such as collagenase and other reagents that disrupt the extracellular matrix and cell–cell connections, often while agitating the solution. This is then followed by vortexing, centrifugation and other steps to aid in the purification of single-cell solutions for downstream analysis. In general, these protocols are sequential, with many prolonged and somewhat tedious manual steps, often resulting in a total processing time of an hour per clinically-sized biopsy sample.

The current standard of preparation techniques for tissue dissociation have been characterized extensively in the literature. Specific approaches has been developed for different tissue types, biopsy sizes, and downstream applications with the objective of producing a homogeneous single-cell suspension devoid of debris, aggregates, or off-target cells. A number of specific protocols can be found in resources such as the Worthington Tissue Dissociation Encyclopedia.

Early attempts to improve upon tissue dissociation protocols for particular tissue types of applications date back to the 1970s with investigation into different proteolytic enzymes and enzymatic adsorption at the cell surface [4]. Research has since

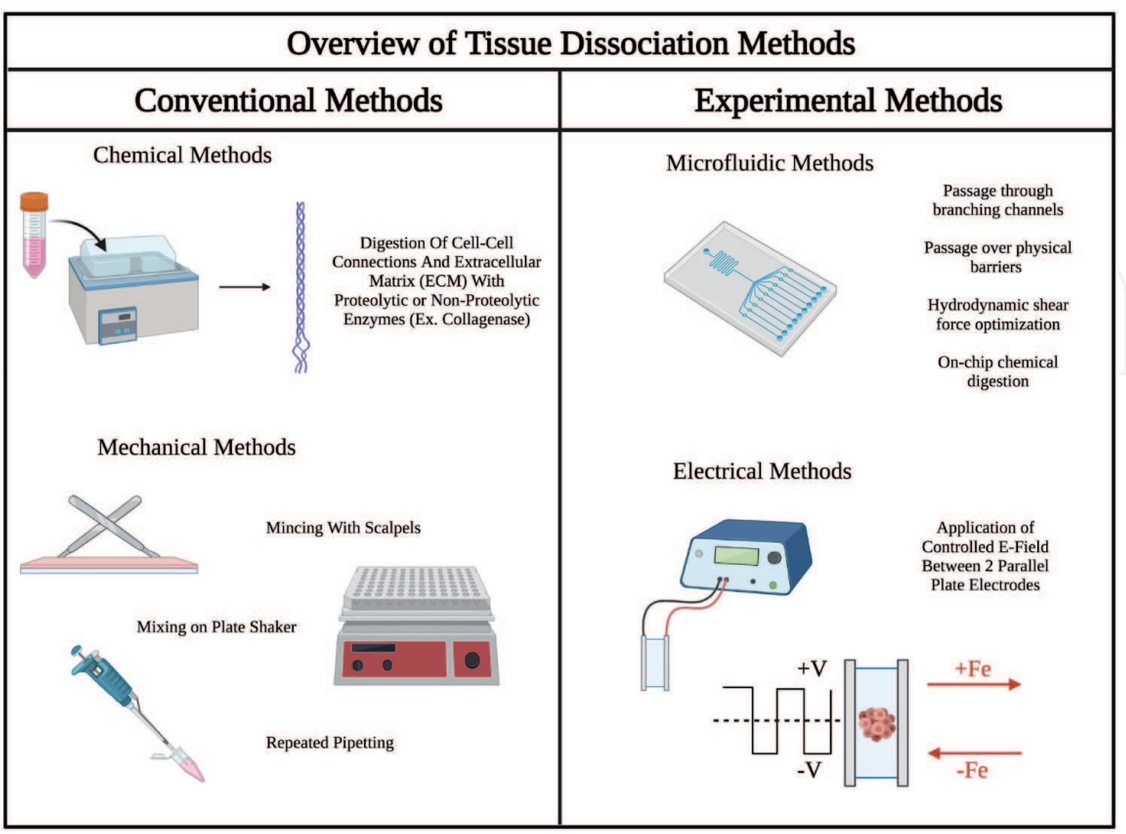

Figure 2.
Overview of various conventional and experimental tissue dissociation methods.

been focused in three main areas: (1) optimizing traditional chemical/mechanical benchtop protocols, (2) translating chemical/mechanical protocols into microfluidic systems, (3) translating chemical/mechanical protocols into commercial sample processing devices (**Figure 2**).

2.2 Optimization of benchtop chemical and mechanical tissue dissociation

Some research has been conducted on the subject of optimizing chemical and mechanical digestion of tissue. As mentioned, both chemical and mechanical dissociation protocols and outcomes are dependent on tissue type. An example of this is illustrated in the work of Robin et al., in which myogenic cells were isolated using collagenase D and dispase II with repeated pipetting, but skin fibroblasts were isolated by collagenase treatment and subsequent mincing [5].

Some research tests numerous enzymes and combinations to determine best chemical conditions for a given tissue. For example, in our recent work, we studied chemical processing treatments and then combined processing with simple mechanical agitation of samples in a 96-well plate on a mechanical plate shaker to determine optimal treatments for dissociation of single cells from bovine liver samples [6]. Physical and mathematical modeling was used to predict optimum mechanical mixing parameters.

While a great deal of research has been conducted in the area of chemical dissociation, comparatively less work has been done on improving benchtop mechanical dissociation. Tissue grinder approaches are a common feature in the literature but are generally not indicated for downstream single-cell analysis due to their tendency to disrupt the cell membrane. Recent work by Scheuermann et al., however, used a tissue grinding benchtop preparation workflow created with downstream SCA in mind [7].

2.3 Microfluidic tissue dissociation

Various microfluidic approaches to the dissociation of tissue and cellular aggregates for recovery of single cells have been investigated by researchers in recent years. Many of the investigated approaches consist of microfluidic chips or processing workflows that use either a purely chemical or purely mechanical approach, but there have also been recent advances which combine chemical and mechanical processing in a single device.

Purely chemical microfluidic approaches to tissue dissociation are present in the literature but are somewhat less explored. An example of a chemically-focused tissue maintenance and dissociation microfluidic device is that created by Hattersley et al., which was used to maintain cellular viability in liver tissue biopsies over 70 hours [8]. In addition to this, the researchers performed an on-chip collagenase digestion.

Other mechanical and chemo-mechanical approaches involve applying force against a physical barrier or using hydrodynamic flow-based disruption to dissociate cellular aggregates and tissues. More recently, both of these methods have been combined into single microfluidic systems for improved results.

2.3.1 Applied force against a physical barrier

A common approach in microfluidic tissue dissociation devices involves the passage of cell aggregates through or over objects to physically disrupt cell–cell bonds. Lin et al., created an enzyme-free microfluidic chip for neurosphere dissociation which used micropillars with 20 μm gaps [9]. When a driving force was applied via a syringe pump, the spheroids were driven through the gaps, separating them into their constituent single cells. Another similar approach is the Biogrid, a silica-knife

microfluidic device created by Wallman et al. for enzyme-free dissociation of stem cell aggregates [10]. The Biogrid knives had edge thicknesses of 30 μm and grid spacing of 200 μm. Another example of physical-barriers in microfluidic tissue dissociation is the microfluidic filter device reported by Qiu et al., which consisted of 2 nylon mesh membranes with 50 and 15 μm mesh sizes [11]. The mesh can be used to both elute single cells and retain cellular aggregates for further processing.

Physical-object based disruption can be an effective method to separate cellular aggregates into smaller aggregates and single cells, and (in the case of mesh) to selectively retain or elute cells and aggregates based on size. However, for these methods to work, there must be an applied force, usually in the form of a syringe pump-generated pressure-driven flow. Additionally, sample loss and cell death can occur with processing, as cells collide with, deform, and stick to or are ruptured by the objects in their path. Sample constituents, such as cell free DNA and cellular debris, can also lead to device fouling, causing significant clogging of narrow apertures through which cells must pass.

2.3.2 Flow-based disruption

Another method for cellular aggregate disruption is passage through a mechanically-optimized dissociating microchannel. Examples often include concepts such as hierarchical or branching microchannels, in which subsequent channels are half the width of prior channels. This principle is used in a set of two papers by Qiu et al., in which a network of branching channels is created and then optimized with shark-fin geometry in order to increase cellular recovery of single cells from aggregates passing through the chip [12]. The channels also used repeated channel expansions and constrictions in order to produce hydrodynamic fluid jets [13], which imparted forces on cellular aggregates, aiding in dissociation into single cells.

Other, previously utilized approaches include placing tissue specimens within a confined area on a chip and applying fluid jets to the tissue core. This concept is present in another paper by Qiu et al., in which the researchers use precision-flows to create a hydrodynamic mincing device [14]. The device accommodates tissue specimens up to 1 cm in length and 1 mm in diameter, focusing shear forces at distinct locations to improve contact between areas of the tissue and enzymes that aid in the disruption of cell–cell adhesion.

2.3.3 Integrated microfluidic tissue dissociation devices

In 2021, a notable work by Lombardo et al. integrated three different tissue processing techniques on a single microfluidic chip – enzymatic digestion, hydrodynamic shear force, and mesh filtration - arguably representing the first chip of this kind [15]. This indicates a progression towards a "Next Generation" of microfluidic tissue dissociation devices. Their system combines tissue digestion, disaggregation, and filtration steps, and was tested on an array of kidney and mammary tissues. Using this technique, 2.5-fold greater recovery of single cells was obtained than with traditional techniques. They also demonstrated recovery of target cell type numbers at particular timepoints during device processing and demonstrated the utility of their device in a single-cell sequencing study. Progress in this area is likely to gain traction in the coming years.

2.4 Commercial tissue dissociation devices

Companies such as Miltenyi Biotec and S2Genomics have attempted to address the need for obtaining single cells from tissues by creating products that automate many of these functions, reducing manual labor and simplifying tissue dissociation

workflows (e.g. the GentleMACS Dissociator). Miltenyi Biotec has made progress in addressing limitations imposed by differences among tissue sources and organs by selling dissociation kits specifically designed for organism (e.g. mouse vs. human) and organ (e.g. small intestine, lung, tumor, brain). Despite this progress, there remains a significant need for improving the size, cost, complexity, and performance metrics of these devices in order to enable improved cancer diagnostics via SCS. Key limitations of the GentleMACS Dissociation system, for example, are high costs in excess of $500 USD for only 25–50 preparations.

2.5 Electrical tissue dissociation

Although it has only been recently characterized, applied oscillating square wave voltages have been shown to aid in the dissociation of complex tissues into their component single cells [16]. Excellent cellular dissociation was observed with 1 kHz electrical conditions in a matter of <5 minutes, significantly faster than conventional chemo-mechanical approaches. The exact mechanism of the observed electric field induced tissue dissociation is not completely understood at this time but may be related to known effects of electric fields on membrane potential, ion transport, cellular movement, and proliferation.

A key finding of this work is the importance of oscillation frequency on tissue dissociation. Higher oscillations in the MHz range have previously been used to decrease heating and electroosmotic effects in electrophoretic applications [17], and previous research has shown that cells can reorient themselves in such electric fields in a manner dependent on the orientation and shape of the cell, as well as the electric field properties [17]. Presumably, the significantly lower dielectric constant of the cell membrane, and higher conductivity than the medium of suspension or cytoplasm can lead to charge accumulation at the membrane, and induced dipole moments that can generate shear stresses within the tissue, leading to alignment, rotation, and stretching behaviors. It is also thought that application of electric field induces a torque on the membrane glycocalyx, which is analogous to an externally applied mechanical force, and results in movement [18].

2.6 Regional dissociation methods

Within tissues, different regions of interest exist with different cellular properties. These regions can often be identified by microscopy or other methods but may be difficult to pick out using conventional heterogeneous sequencing approaches. Approaches such as manually excising an area of interest in a tissue sample for later analysis can still include numerous off-target cells.

Selective dissociation of cells from specific target regions of interest has been an area that has fascinated scientists since the 1980s. In the first work of its kind, Freyer and Sutherland selectively dissociated and characterized cells from various regions of tumor spheroids by applying a dilute trypsin solution at 18 to 30 degree angles in custom dishes [19]. Freyer and Schor later characterized the dissociation of cells from different spheroidal regions using an automated device that exposed outer spheroidal layers to 0.25% trypsin [20].

More recent advances in regional dissociation and regional tissue interrogation have also been made. The most notable technique in the field of region of interest analysis is arguably laser capture microdissection, a technique that is integrated with common optical microscopy infrastructure and used with conventional tissue-on-slide imaging. This technique uses optical interrogation to manually or automatically select tissue regions of interest and separate them from the surrounding tissue, placing targeted cells in a tube for subsequent molecular analysis. Other

ROI techniques such as Computer-Aided Laser Dissection can incorporate machine learning and other informatics techniques into the workflow for automated computer recognition of regions of interest for subsequent selection.

2.6.1 Laser capture microdissection

The laser capture microdissection (LCM) process dates back to high-profile works published in *Science* in 1996 and 1997 by Emmert-Buck et al., and Bonner et al., respectively [21, 22]. The IT-based workflow begins with an initial microscopic visualization, followed by selection of cells of interest on a conventional microscopy slide. A region of interest (ROI) is then manually selected in conventional LCM workflows. After ROI selection, a cap covered with transfer film is lowered onto the tissue surface. A laser pulse is then activated, which in turn activates the transfer film, causing the cells in the selected area to adhere to the film on the cap. In the UV-based LCM technique, the laser is able to cut around the cell itself, bypassing the plastic adhesion process [23]. The selected cells can then be placed into a tube for molecular analysis and other downstream tests. The technique can also be used as an alternative to histological staining in proteomic sample analysis [24]. To date, combination of dissociation of the selected tissue for SCA has received only scant attention, but further research in this area over the next few years would represent a logical progression of existing microdissection technology (**Figure 3**).

2.6.2 Computer-aided laser dissection and machine learning

In the years since the creation of the LCM technique, many researchers have recognized a key limitation in the lack of automation of LCM: the requirement for manual selection. This results in low-throughput, making processing large amounts

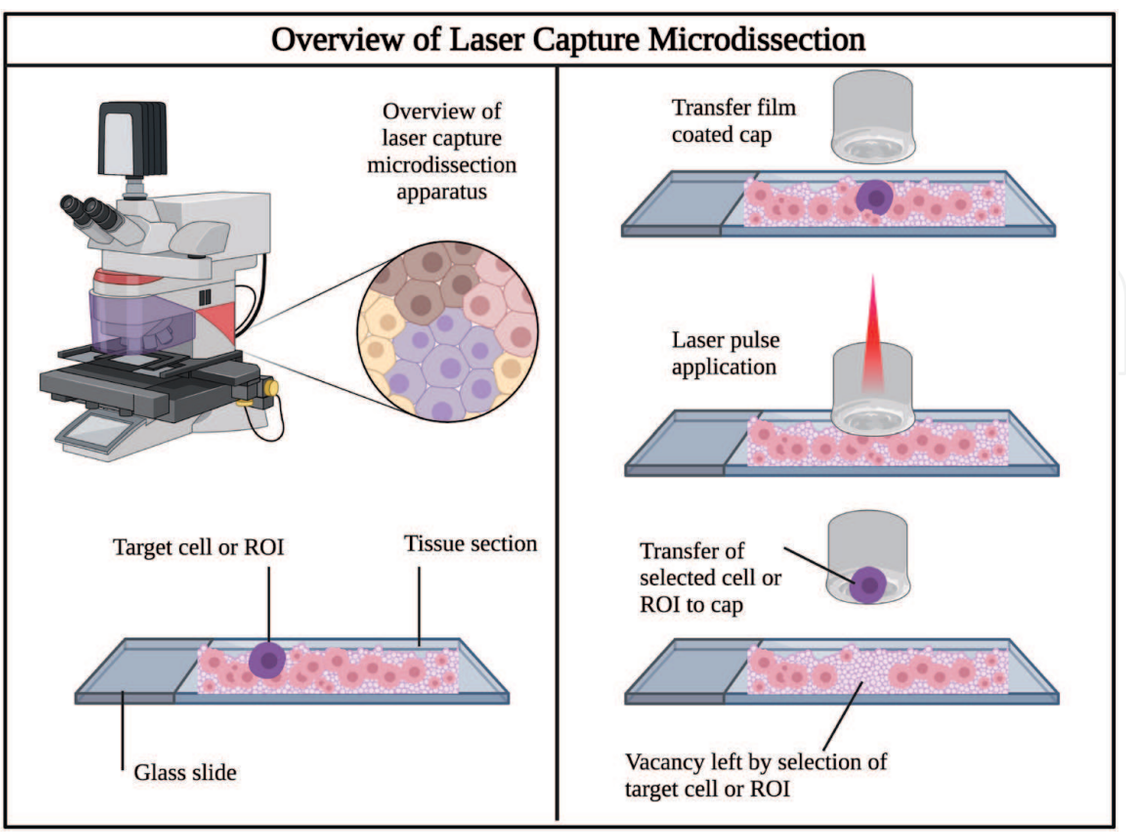

Figure 3.
Overview of laser capture microdissection.

of tissue samples a difficult task. A proposed solution lies in the Computer-Aided Laser Dissection (CALD) technique, which uses algorithms to recognize areas of interest and oversee dissection in an automated or semi-automated manner.

Statistical learning methods have long been proposed as a tool for region of interest detection in cancer tissue specifically, but have also been widely used in the interpretation of X-rays and other biomedical image data for diagnostic purposes [25, 26]. In recent work by Hipp et al., authors compare use cases of integrating image analysis tools into the LCM workflow [27]. They discussed possibilities of integrating existing software like ImageJ, as well as techniques such as spatially invariant vector quantization and probabilistic pairwise Markov models. Recently, Ren et al. have translated a machine learning technique to achieve automatic partitioning of selected regions of interest [28]. They used a K-means clustering algorithm with a fuzzy c-means clustering algorithm to discern ROI, and automatically selected clustering results. It is likely that this particular area will experience significant growth in the coming years.

A major limitation of computer vision and machine learning techniques for region of interest detection in tissue samples is the need for algorithm training. Additionally, different algorithms must be used to detect regions of interest in different tissue types, as regions of interest can have distinct visual morphologies across tissues. Furthermore, these techniques may be poorly equipped to deal with irregular appearances of regions of interest, which could pose a hurdle towards their translation in cancer diagnostics.

3. Single-cell suspension purification methods

After tissue dissociation into cellular suspensions, workflows must be created that enable selection of single-cell populations for downstream analysis. A critical step in getting purified single-cell suspension lies in the discernment of single cells from large cellular aggregates and debris from the extracellular matrix and lysed cells.

We herein provide an overview of methods for removal of cellular clumps and debris, a sample preparation step which has become known as purification. These procedures are unique from cell sorting and manipulation methods due to the nature of their position in the pipeline from tissue to SCS. While cell sorting and manipulation methods are occasionally equipped to separate single cells from doublets, for example, they generally cannot be used without an intermediate purification step when analyzing dissociated tissue samples.

These sample purification processes are distinct from target cell selection methods as they are not focused on separating rare target cells from heterogeneous suspensions but are instead focused on simply obtaining purified single-cell suspensions themselves. While it has been shown that techniques such as flow cytometry facilitated FACS can be used directly from dissociated tissue samples, this section will focus on conventional methods of purification. Methods that will be discussed here are filtration and chemical debris removal (**Figure 4**).

3.1 Filtration

Filtration techniques are currently considered to be the gold standard for the sample purification step. Commercially available mesh filters can be purchased and incorporated into the benchtop preparation workflow when processing tissue into single-cell suspensions for SCS. These commercial filters, created by companies such as Miltenyi Biotec, are often sold as “caps” that fit directly into centrifuge tubes. The tissue dissociate is simply poured over the cap, and filtrate passes through into the bottom of the tube. While this represents an example of

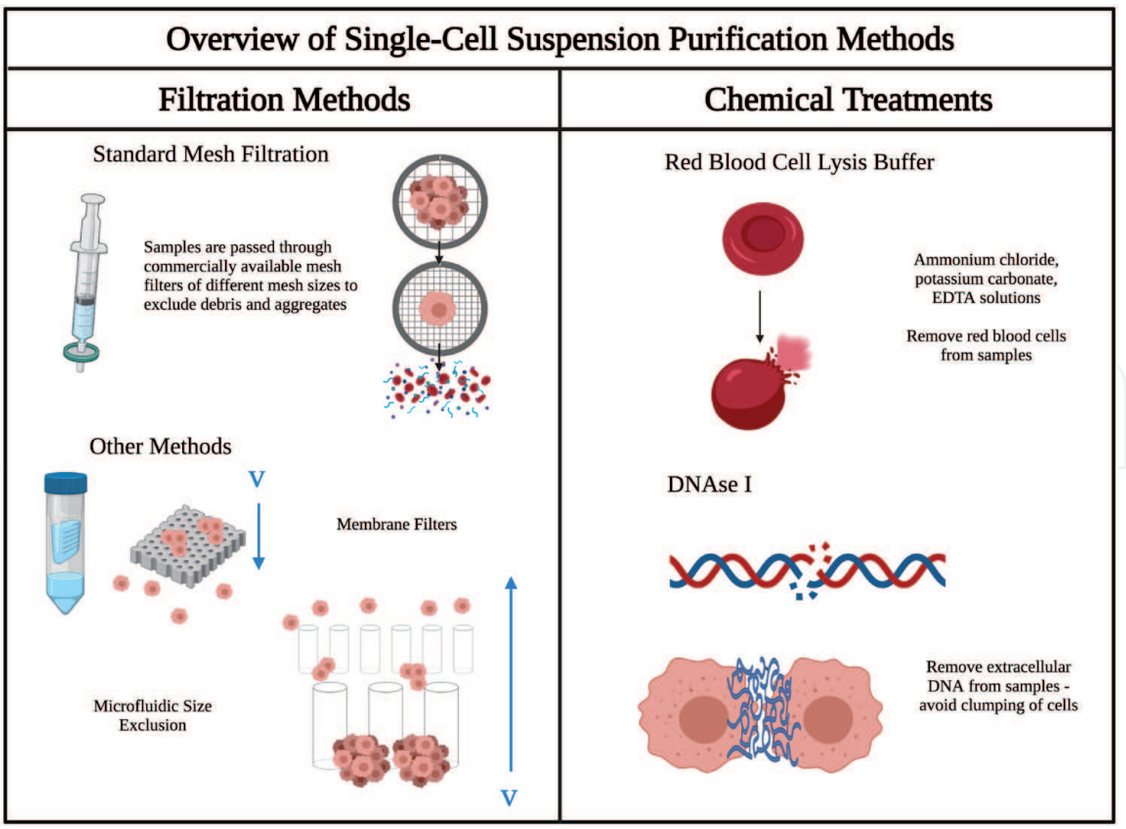

Figure 4.
Overview of single-cell suspension purification methods.

conventional filtration, other filtration methods can also be used. Different filter types include membrane filters, post filters, and weir filters.

Membrane filters contain porous structures that can be used to trap larger cells, such as tissue cells, while excluding smaller cells, such as red blood cells. Pillar based filters and weir filters all consist of obstacles about which cells must navigate. Weir filters are characterized by a singular obstruction nearly closing off the channel, permitting only particles small enough to navigate the gap through, while pillar filters consist of numerous evenly spaced pillars. These systems are all prone to clogging with debris and clumps of tissue, especially at high cellular densities, although systems which use dynamic filtration instead of barrier filtration can clog less. Forward and reverse flow can be used to unclog filters if they are incorporated into a microfluidic environment, but clogging still poses an issue, nonetheless.

Other filtration techniques of interest include cross-flow filtration and size exclusion filtration. Cross-flow filtration is a filtering technique that has been used in the purification of animal cells since the 1990s [29]. It offers a prospective solution to one of the most ubiquitous issues of all filter-based cellular purification systems, clogging, by using sieve-like sorting. Size exclusion filtration can be created with progressively decreasing filter mesh sizes but is occasionally used to refer to pillar-based filters containing tiered post arrangements with decreasing gaps. These systems can be translated into microfluidic chip formats in order to isolate differently sized particles in different regions of the chip [11, 30].

3.2 Chemical methods for debris removal

In addition to mechanical filtration of dissociated cellular suspensions, chemical methods for debris removal are also highly utilized. Common methods include removal of red blood cells, and removal of extracellular DNA.

In the SCS workflow, non-nucleated red blood cells are often excluded from analysis by chemical treatment with products such as red blood cell lysis buffers that are commercially available for purchase. These buffers can contain ammonium chloride, potassium carbonate, and EDTA. The solution is incubated on pelleted dissociated tissue for 4–5 minutes at room temperature, and then deactivated by adding PBS, and subsequently removed, according to BestProtocols guidelines provided by ThermoFisher Scientific.

Extracellular DNA removal is also a popular purification step for some SCA protocols. During the tissue dissociation process, extracellular DNA is commonly released. The DNA sticks to the cells, causing them to form aggregates. This can cause aggregation on filters, which can lead to sample loss and clogging. DNAse I solutions are a proposed answer to this problem. They are recommended to be applied to solutions at a concentration of 100 μg/mL, according to BestProtocols. An issue with DNAse I use is that it is inhibited by actin release from dead cells. It also must be removed from the sample for successful downstream analysis with SCS.

4. Cell sorting and manipulation methods

After the sample purification step, many single-cell analysis workflows require additional processing steps to eliminate any remaining doublets or off-target cells. Therefore, some SCS workflows are concerned not only with the selection of individual single cells, but also the retrieval of specific subsets of cells from heterogeneous cellular populations. Examples include selecting for cancerous cells from a predominantly healthy cell population or selecting for cells with rare genetic mutations. Sorted suspensions of cells of interest are then able to enter the remaining part of the SCS workflow, consisting of lysis and analysis steps.

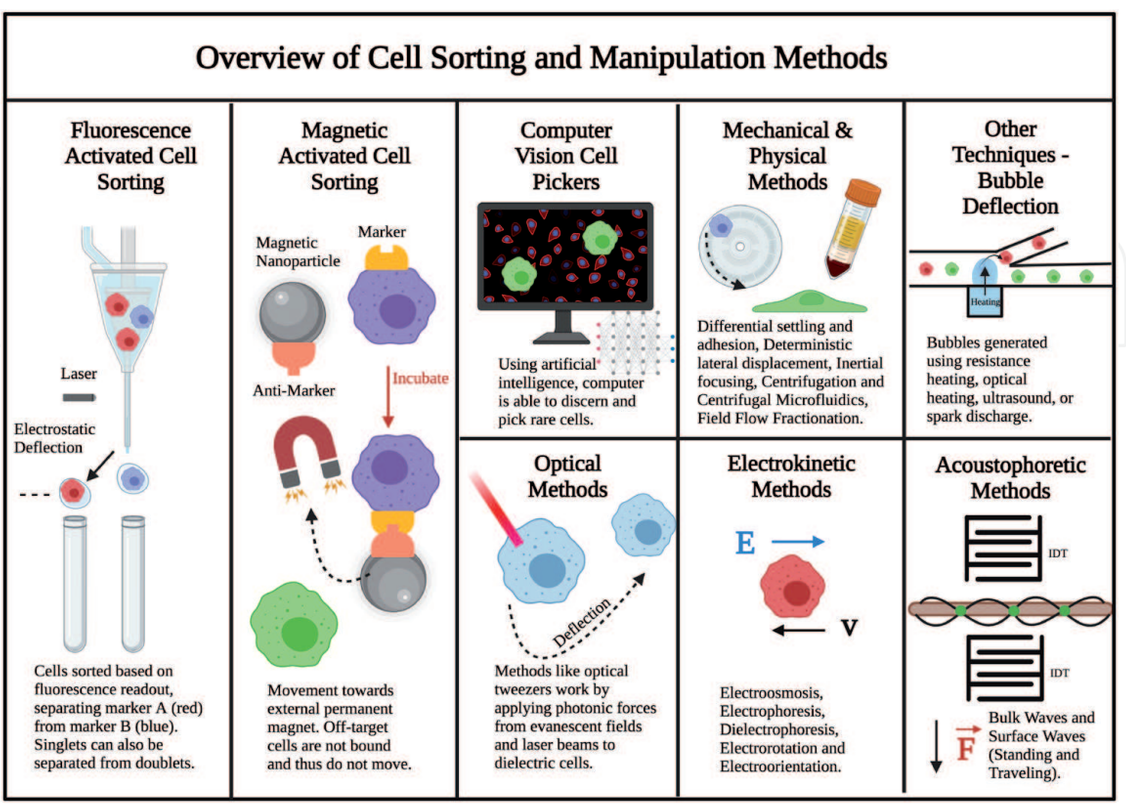

Figure 5.
Overview of cell sorting and manipulation methods.

Methods that will be discussed in this section cover techniques for cell sorting and manipulation, many of which have relevance to target cell selection. Topics to be discussed include fluorescence activated cell and droplet sorting, magnetic activated cell sorting and magnetophoresis, computer vision and cell picking techniques, optical techniques such as optical tweezers and focused beams, mechanical and physical cell sorting, electrokinetic and acoustophoretic cell sorting (**Figure 5**).

4.1 Fluorescence activated cell sorting

4.1.1 FACS history and commercial flow cytometry

Fluorescence Activated Cell Sorting (FACS) is a ubiquitous technique created in the late 1960s by "Bonner, Sweet, Hulett, Herzenberg, and others" [31, 32]. The technique built upon previous cell sorting developments in flow cytometry and is currently used widely by researchers in order to obtain cellular suspensions of target cells.

In the conventional FACS process using a standard flow cytometer, a cellular suspension sample is injected through a fluid sheath into a flowing stream in laminar flow conditions. The cellular suspension is previously treated with a fluorescent probe or antibody, which can be either a general probe, or a probe specific for a particular cell type or characteristic of interest.

A laser illuminates the stream directly before droplet formation, and fluorescence emission scatter information is transmitted to a detector. Immediately afterwards, aerosolized droplets are produced by vibration of the instrument, and contain either a single-cell or an absence of cells, as verified using the recorded fluorescence signal information. The droplets are charged based on cellular contents and are then electrostatically deflected into containers based on charge. Uncharged droplets maintain their original course.

Since the invention of FACS, numerous technological improvements have been developed, such as incorporation of multiple fluorescent lasers and detection capabilities within commercially available flow cytometry instruments [32]. FACS techniques are constantly evolving, and have also notably been translated outside of the flow cytometry workflow and into microfabricated microfluidic chip systems over the past two decades [33, 34]. This translation can circumvent limitations of the traditional aerosolization process while still maintaining the general principles of FACS such as real-time sample classification and binning. While traditional FACS is an open-system and lacks sterilization, microfluidic FACS chips are sterile and single use.

Other persistent limitations of FACS are relatively high cell number requirements ($>10^6$ cells) and reagent consumption, high cost of flow cytometry instruments, and the potential for cross-contamination, and clogging, especially when analyzing dissociated tissue samples [35].

4.1.2 Fluorescence activated droplet sorting

Fluorescence Activated Droplet Sorting (FADS) is a microfluidic fluorescence-activated sorting technique which uses many of the same principles of FACS [36]. FADS was first described by Baret et al. in 2009 as a method that combined the benefits of FACS with those of microtiter-screening. The process involves encapsulation of single cells within emulsion droplets, which are then dielectrophoretically sorted as in FACS. In Baret et al.'s initial work, sorting rates of up to 2,000 droplets/second were obtained with a false positive rate of $<1/10^4$ droplets. Limitations were seen in co-encapsulation of fluorogenic substrate with cells, not in sorting. Errors

could be minimized at low sorting densities (<10^5 cells), which are unable to be run using conventional FACS flow cytometry [37]. Ever since this initial work, FADS has been used in numerous single-cell applications, and other applications, such as synthetic biology. Newer systems have focused on increasing throughput, and multiplexing droplet sorting capabilities [38].

4.2 Magnetic activated cell sorting and magnetophoresis

Magnetic Activated Cell Sorting (MACS), or magnetic separation of target cells is one of the most commonly used separation techniques for cellular selection. In this technique, nanosized superparamagnetic beads are bound to a particular antibody or other recognition element for a specific cellular target of interest. Specifically bound cells are then removed from solution using a magnet, followed by washing of the beads and liberation of the bound cells. Common cellular-recognition elements include the Epithelial Cell Adhesion Molecule (EPCAM) for separation of CTCs from whole blood, such as is used in the commercially available Veridex Cellsearch system and other applications [39]. Other approaches can use separation via specifically engineered indirect interactions, such as biotin/streptavidin coupling.

Once nanoparticles are co-incubated with cellular solutions, they can bind to target cells through antibody/antibody, antibody/antigen, or other interactions. The bound target cells can then be separated from unbound off-target cells and collected by moving them to a different location within a microfluidic chip or tube using an external permanent magnet, via a process known as "magnetophoresis". Electromagnetic coils can also be used to enact magnetophoresis, although this is a less common approach. Researchers such as Pirozzi et al. have used this technique in combination with microfluidic oil/water immiscible phase filtration in order to separate cancer cells from various sample matrices, including unprocessed whole blood.

MACS principles have been translated into commercially available systems. The first commercially available MACS system was created by Miltenyi in 1990 [40]. CTC enrichment of 10^5 fold was obtained using high magnetic gradient column MACS, but purification was found to be lower than would be clinically applicable in CTC work [35, 41]. This has since been improved using magnetic sweeping and other techniques.

Much work on MACS is focused on CTC isolation from blood using markers, a much easier problem to solve than isolation of target cellular populations from dissociated tissue samples. However, some work has been conducted on stem cell isolation of solid-tissue originated cells [42]. Other work has been conducted on MACS separation from tumor tissue, but there has been little progress in this area as MACS separation is not a label-free technique, and relies on marker-based separation of specific target cell types [43]. As such, MACS is not considered to be an appropriate technique for understanding the true extent of tissue heterogeneity but can be used within the processing workflow to isolate particular cell types of clinical interest with known unique extracellular markers.

4.3 Computer vision and robotic cell picking techniques

Automated cell picking instruments are recent commercially translated instruments which can take various forms. Common themes in cell pickers are the use of optical imaging and computer vision to distinguish cells of interest. Certain cell pickers, such as the Shimadzu Cell Picker, are focused on picking cell colonies off culture plates for use in cell line construction and cloning applications. Other systems are designed specifically for isolating rare cells from liquid biopsies, such

as the CellCelector instrument, an Automated Lab Solutions product. Many are designed specifically with downstream single-cell genetic analysis in mind.

Cell pickers can use diverse methods to select cells of interest, such as image recognition of fluorescent cells, distinct cellular morphologies, and more. After selection occurs, cells must be physically "picked", or removed. Most cell picking devices work by using a mechanical micro-manipulation robotic picking system. For example, the Shimadzu Cell Picker works by simply lowering a pipette to a particular location, as ascertained using the automated picking system. The CellCelector is equipped with different picking modules using capillaries, metal scraping tips, or plastic tips. In addition to translation into commercially available instruments, computer vision and robotic sorting have also been incorporated into microfluidic cell sorting [44]. Similarly to LCM, robotic cell picking techniques are often lower-throughput in comparison to other methods (e.g. FACS), despite automation. They are also frequently performed in open-air, posing a contamination risk.

4.4 Optical techniques

Optical techniques can be used in order to physically trap cells in suspension using elements such as focused laser beams, fields, and more. Some commonly used optical sorting techniques include optical tweezers and focused optical beams. The optical tweezers technique is arguably the most well-characterized technique and can be used to noninvasively move and separate dielectric particles in solution, such as cells, in a label free manner. Optical beams use radiation forces to achieve cell movement, and can also use optical radiation for other applications, such as bubble-induced cellular deflection.

4.4.1 Optical tweezers

Optical tweezers, invented by Ashkin et al., in 1986, are used in the noninvasive label-free separation of cells [45]. They work by applying photonic forces from evanescent fields and laser beams. Depending on properties of the refractive indices, cells and other dielectric particles are acted on by forces that can hold them in place, or move them to a new location [46, 47]. Optical tweezers are highly sensitive instruments that enable applied force resolution in the piconewton range, with sub-nanometer spatial resolution. Thus, these are highly useful tools in the microfluidic separation of individual target cells.

In addition to their usefulness in sorting, optical tweezers have been applied to the interrogation of numerous biological questions from assessing cellular responses over time to analyzing cellular biomechanics [46, 48]. While they have been applied to the separation of embryonic stem cells and tissue engineering, they have yet to be comprehensively utilized within the tissue to single-cell workflow [46, 49, 50]. Robotics technology has also been integrated with optical tweezers in recent years [51–53].

4.4.2 Optoelectronic tweezers

Optoelectronic tweezers (OETs) combine optical manipulation with electronic capabilities, specifically electrode-based DEP, to create a promising label-free technique that is frequently higher-throughput than optical tweezers, and a potentially promising area for SCS sample preparation. Optical images are projected onto a surface, creating temporary visual electrodes, which are used to locally apply an electric field, producing a dielectric force which can be exerted on the cells. In the event that the cell is less polarizable than the liquid, such as with cells in culture media, the

pattern will repel it, which can be used to confine a cell to a specific location while fluid and other materials flow freely [54]. In addition to OETs potential for trapping, cells can also be transported through the chip and sorted via dielectrophoretic forces via the creation of light-defined channels and use of size selective sorting, respectively [55].

The translation of optoelectronic tweezers is somewhat limited by high required optical intensities, as is also a key limitation of standard optical tweezers [56]. However, light intensities have been used for cell separation in OET devices that are 100,000-fold lower than optical tweezers [57]. Other advances, such as combination with microfluidics and electric field re-orientation, have been used to increase the potential for OET translation. OETs have been used to manipulate mammalian cells in a variety of ways, from replicating the tumor microenvironment on a microfluidic chip [58] to their combination with scRNAseq preparatory microrobotics [59].

4.4.3 Focused optical beams

Focused optical beams can be used in order to apply radiation force to individual cells in a microfluidic environment, resulting in precisely controlled cellular manipulation, similar to that observed in the optical tweezers technique [60]. Cells can be deflected into an alternative channel at a junction with the influence of a laser pulse [61].

Focused optical beams such as lasers can also be used in cell sorting in an additional way. Localized heating from laser light can result in bubble formation, which can in turn produce mechanical deflection of target cells [62]. Optical scalpel techniques can also be used in order to assess different cellular components, but these techniques are not in-line with the expected workflow for downstream single-cell analysis.

4.5 Label-free sorting by mechanical and other physical properties

Other methods can sort cells based on their physical properties such as density, size, and settling behavior. Techniques that will be discussed here include settling and adhesion-based techniques, deterministic lateral displacement, inertial focusing, centrifugal microfluidics, and field flow fractionation.

4.5.1 Settling and adhesion

Different cell types settle and adhere differently in *in vitro* environments, due to a combination of material interaction and mechanical properties. Bailey-Hytholt et al. recently demonstrated effective rare cell selection via different settling behavior of cells in polystyrene wells [63]. This approach was used in a prenatal testing workflow for the purpose of separating rare extravillous trophoblasts from infants *in utero* from maternal cervical cells in cervical swabs. Bailey-Hytholt also notably investigated trophoblast enrichment via differences in surface adhesion on an inclined plane [64]. The principles of differential adhesion have also been applied to embryonic cell sorting [65].

Differential surface adhesion can also be artificially engineered by coating a patterned surface on a microfluidic chip with a particular marker or ligand. Stott et al. were the first to take this approach to cellular isolation in 2010 using a Herringbone chip coated with EpCAM to detect CTCs in the blood [66]. EpCAM has also been used in other more recent approaches [67] and EpCAM-free approaches to cancer cell isolation have also been investigated by looking at specific peptides of interest for particular cancer types [68]. However, these approaches are not label-free, which significantly limits their translational potential into the tissue to SCS NGS workflow.

4.5.2 Deterministic lateral displacement

Deterministic lateral displacement (DLD) was initially used for particle separation in 2004 in Huang et al.'s notable work [69]. Since then, it has been widely used for cell separation, as characterized in detail elsewhere [70, 71]. Deterministic lateral displacement structures have been used in the label-free separation of large cells for tissue engineering [72], and cancer cells from diluted whole blood samples [73]. This method uses different size-dependent hydrodynamic forces to separate cancer cells from red blood cells and other cells as they pass through a channel equipped with numerous posts through which the cells must navigate. Various works have also shown that triangular posts can help to optimize results [73, 74].

A similar approach to DLD is deterministic cell rolling. Cell rolling devices consist of ridged microfluidic channels, which, like DLD, enter the flowpath of cells, resulting in mechanical deflection. The surface of the channel can be modified with a target of interest, such as P-selectin, which can briefly bind target cells, deflecting them to a different location within the chip [75].

4.5.3 Inertial focusing

Inertial focusing is another technique frequently used to sort cells in microfluidic chips by inducing movement across laminar flow streamlines. Continuous inertial focusing, ordering, and separation of particles was demonstrated by Di Carlo et al. in 2007, and has since been applied extensively in microfluidic cell separation [76]. Di Carlo et al. demonstrated that particles in laminar flow conditions can migrate across streamlines in a "continuous, predictable, and accurate manner". The migration is caused by particle lift forces in conditions where inertial flow is significant. Boundary effects at microchannel walls can create this lifting phenomenon. Particular channel geometries create inertial forces that result in a bias towards certain equilibrium positions, a phenomenon which can be used for ordering of cells within a given microfluidic chip. Inertial focusing has been used, for example, in spiral microchannels and serpentine channels [77–82]. Inertial effects can also be combined with other hydrodynamic techniques, or flow fractionation techniques as well, such as pinched flow fractionation.

4.5.4 Centrifugation and centrifugal microfluidics

Centrifugation techniques are some of the oldest methods historically used in the separation of various blood components, such as nucleated cells and red blood cells. This same principle has been used in the sorting of CTCs from other cells in blood and bone marrow samples [83]. While developments using standard tube-based centrifugation methods have been minimal in recent years, centrifugal microfluidics has spawned a new field of target cell separation research.

Centrifugal microfluidics techniques have been investigated for over 40 years [84]. Techniques have been created using recycled or repurposed compact disks or disk-shaped polymer-based materials, which are rotated around a conventional or custom centrifuge to separate rare cells from other sample components. These systems are referred to as Lab-on-a-disk or Lab-on-a-CD platforms [84]. Snider et al. recently created one such system for application in the separation of single cancer cells from dissociated biopsy cores [85]. They were able to observe up to 80% capture efficiencies when using 2,000 to 15,000 cell input samples. While some centrifugal microfluidics applications perform chemical

and mechanical cell lysis on chip, this system preserved cells in-tact for visual microscopic analysis. Centrifugal microfluidics can be integrated with geometric, magnetophoretic, and dielectrophoretic separation approaches [86].

4.5.5 Field flow fractionation

Field flow fractionation (FFF) and, in particular, sedimentation field flow fractionation (SdFFF) is a label-free method that can be used to separate various cell types. While FFF is primarily utilized in the separation of subcellular particles, macromolecules and nanoparticles, SdFFF has been used in the separation of stem cells [87]. For example, SdFFF has been used in the isolation of cancer stem cells from colorectal cancer cell lines [88]. Pinched flow fractionation (PFF) is a similar technique, but it is mainly used in separation of blood components and non-mammalian cells [89, 90]. FFF techniques have also been combined with other techniques, such as dielectrophoresis [91, 92].

4.6 Label-free sorting by electrokinetic properties

With the exception of electrical tissue dissociation and FACS, electrokinetic methods for cell separation and recovery have heretofore not been discussed in this chapter. Electrokinetics are physical effects resulting from applied electric fields that produce physical movement of cells and other particles [93]. While our discussion of FACS mentioned electrostatic sorting of charged droplets, in this section, we will discuss electroosmosis, electrophoresis, and dielectrophoresis within the context of cellular separation from dissociated tissue samples. We will also briefly discuss electrorotation and electroorientation phenomena (**Figure 6**).

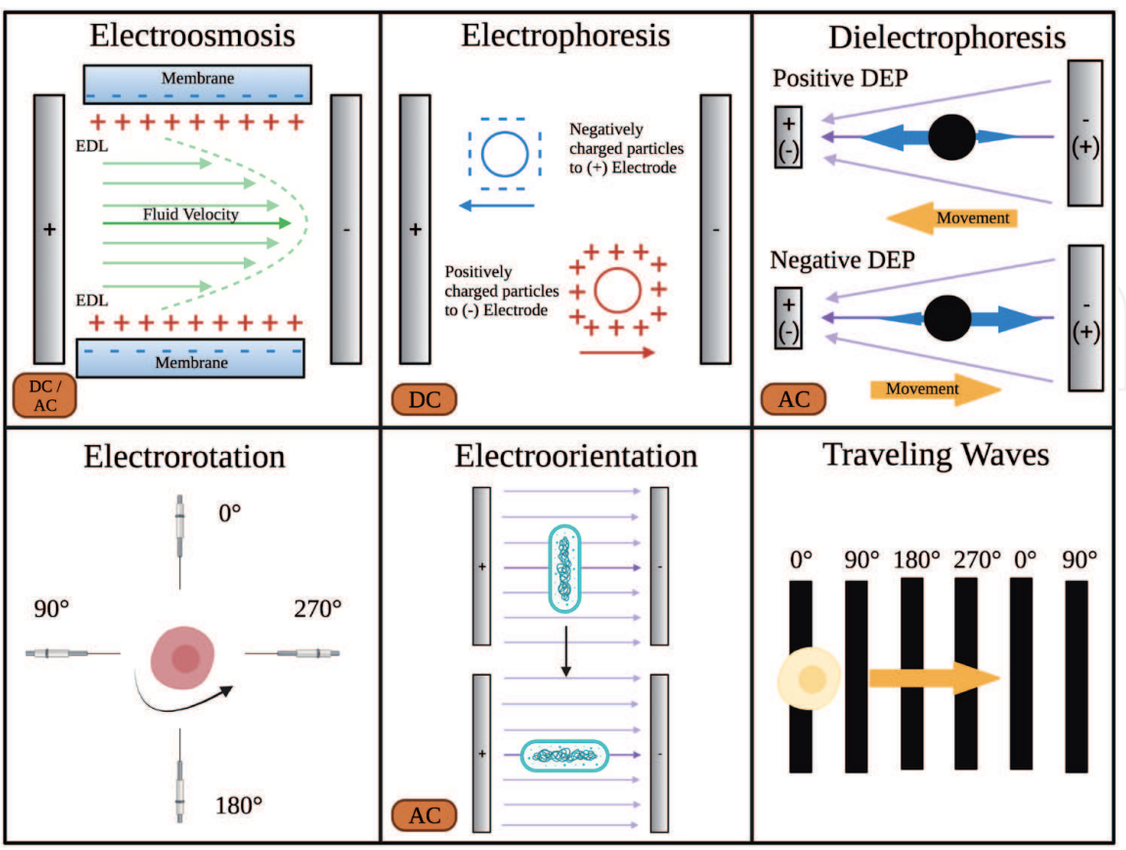

Figure 6.
Overview of electrokinetic phenomena used in the manipulation of cells.

4.6.1 Electroosmotic flow

Electroosmotic flow moves fluids and the particles suspended within them by induced migration of solvated ions [94]. Therefore, electroosmosis is characterized by fluid movement, as opposed to particle movement within a fluid, the mechanism at play in electrophoresis and dielectrophoresis. Electroosmosis has also been used in combination with FACS in order to sort fluorescent cells. Electroosmotic flow has also been used to create microfluidic on-demand particle separation by inducing a vortex on the conductive surface to direct particles, such as cells, towards an outlet. This phenomenon is referred to as controlled electroosmotic micro-vortexes or fluid pumps [95, 96]. Alipanah et al. have recently used induced charged electroosmotic flow and magnetic fields in conjunction, and managed to use flow vortexes to enable the magnetic field to overcome particle drag forces [97]. The effect can be tunable, as demonstrated by Yan et al., by adjusting modulating electrode and insulating post thickness, which correspondingly increases flux within the channel [95].

A major limitation of certain electroosmotic protocols lies in the use of DC electric fields. DC fields can induce electrolysis of water, bubbling, and production of reactive oxygen species, which can decrease viability of cells and introduce turbulence. In order to overcome these limitations, AC electroosmosis has gained some attention. For example, Puttaswamy et al. used AC electroosmosis to avoid these negative effects of DC field application [98].

4.6.2 Electrophoresis

In electrophoresis, particles suspended within a solution move towards an electrode with opposite charge in a DC field with a force proportional to their surface charge. Cells have a slightly negative charge, resulting in their migration towards the positively charged electrode. While electrophoresis is generally considered to be less efficient in the sorting of cells when compared to other processes such as dielectrophoresis, it has still been applied in the separation of mammalian cells [99]. One approach to mammalian cell separation via electrophoresis is with capillary electrophoresis, which can be coupled with an inkjet printing system. This approach was used to separate HUVEC, HepG2 and Caco-2 cell lines in a drop-on-demand technique by Zhang et al. [100]. Water-in-oil continuous flow techniques with encapsulated cells can also be used to improve electrophoretic droplet sorting of single cells from doublets [101]. However, most uses of electrophoresis are in the detection of proteins, antibodies, and other cellular constituents.

4.6.3 Dielectrophoresis

Dielectrophoresis (DEP) is different from electrophoresis in that cells move in a non-uniform field as a result of their polarizability, not their charge. Non-uniform AC fields induce dipole moments across cells, effectively polarizing them and inducing migration. If the cells have a higher permeability than the fluid they are immersed in, they are attracted to the area with the highest field intensity, known as the maxima, in a process called positive DEP (pDEP). If the reverse is true and the cells have a lower permeability than the surrounding fluid, negative DEP (nDEP) will occur, and the cells will migrate away from the region of highest field intensity. The forces of dielectrophoresis depend on cellular properties such as size and shape, as well as the characteristics of the surrounding fluid and electric field.

DEP can be used to create cell traps via the application of a field gradient across planar electrodes. DEP traps are effectively strong enough to maintain cells in a desirable position despite external forces, such as fluid movement. Because

different cell types have different dielectric properties, DEP can be used in the isolation of particular cell types of interest from heterogeneous cellular populations. DEP can also be used to separate cells and aggregates of different sizes within homogenous cellular populations [102–105].

While DEP has also been applied to numerous applications concerning blood, DNA, microorganisms, and other cells, this concept has not been as extensively investigated in cellular suspensions from dissociated tissues [106, 107]. However, initial progress has been made in the creation of a DEP microfluidic device using direct current to trap individual cells from cellular mixtures containing MCF7 and MDA-MB-231 cancer cells, as well as PBMC blood cells [108]. DEP devices can be structured so as to control the DEP at individual locations in a microfluidic chip, such as within specific wells. Lateral DEP devices can be used to position cells using interdigitated electrode arrays [109]. Feedback on cellular capture and location can be assessed by measuring changes in impedance as cells pass through different chip locations.

In addition to pDEP and nDEP, different subtypes of DEP exist, such as closed or conventional dielectrophoresis (cDEP) and traveling wave dielectrophoresis (twDEP) [110]. DEP is also widely multiplexed with droplet microfluidics, optical readout systems, and more, making it a very diverse and versatile technique.

4.6.4 Other electrical phenomena

Other electrical phenomena exist that can be helpful tools in single-cell manipulation and sorting, which could be potentially usefully integrated into single-cell processing workflows from tissue starting samples. The two most important examples of such phenomena are electroorientation and electrorotation.

In electroorientation, cells orient themselves so that they are aligned in a certain confirmation along with an applied electric field. This phenomenon occurs if the induced dipole moment along a particular axis of the cell is stronger, as the dipole moment has varying frequency dependency depending on the axis [17]. This effect is generally seen at higher frequencies, on the order of MHz, and is most frequently seen in non-human cells, although the principle has been applied in DEP systems to human cells as well [111].

In electrorotation, cells are able to be rotated by surrounding electrodes. The principle was first developed in Arnold and Zimmermann in 1988, in order to perform dielectric measurements on individual cells [112]. In this process, surrounding electrodes can be placed at the 0°, 90°, 180°, and 270° positions. The electrodes can emit constant electric fields, but generally use rotating electric fields that are phase-shifted by 90 degrees [17, 112]. As with electroorientation, this is a frequency dependent process.

4.7 Label-free sorting by acoustophoretic properties

Acoustophoresis is a process in which acoustic pressure waves are able to result in movement via standing or traveling wave formation. Acoustic techniques have been combined with microfluidic systems in recent years, creating a new subcategory of "acoustofluidic" devices. Acoustofluidic systems have been used extensively in cell sorting applications and offer benefits such as rapid movement over nm to mm length scales. Different acoustic waves that are used for this purpose consist of bulk standing waves, and surface acoustic waves (SAWs). SAWs are further subdivided into standing SAWs (SSAWs) and traveling SAWs (TSAWs). The key difference between bulk waves and standing waves are that bulk waves, generated by a piezoelectric transducer, propagate through the bulk of the microchannel chamber itself, while surface acoustic waves, generated by an interdigitated transducer,

propagate through the bottom surface of the channel [113]. Acoustofluidic systems are frequently used to sort cells via deformability or other elastic properties.

4.7.1 Bulk standing waves

The first instance of bulk standing wave sorting was characterized by Johansson et al. in 2009, in which an ultrasonic transducer was used to facilitate microfluidic FACS [114]. In the bulk standing wave acoustophoretic process, pressure waves that are identical in terms of oscillation frequency and magnitude are applied to a fluid media in opposite directions, resulting in standing wave formation within the media. Standing waves contain nodes and oscillating antinodes, which represent 0% pressure fluctuation and 100% pressure fluctuation, respectively. In bulk standing waves, the transmitted acoustic wavelength matches the configuration and dimensions of a given channel, and particles are able to move outward. When placed in physiologically relevant buffers such as media, cells are able to be focused into areas within the microfluidic channel via this process as a result of their higher density than the surrounding fluid. They can be separated by size due to differences in forces that dominate at different particle sizes. Specifically, acoustic radiation dominates in larger particles while drag forces induced by acoustic streaming dominate in smaller ones [113].

The bulk acoustic wave separation technique has benefits over other acoustic techniques due to the increased flexibility in transducer placement, which makes microfluidic chip construction less complex. Bulk wave systems tend to operate using a comparatively lower frequency and longer wavelength, which gives them utility in the manipulation of particles of up to 200 μm, such as water-in-oil droplets [115]. Bulk wave mechanisms often require more energy than surface wave methods to facilitate cell sorting, due to the need to propagate signal throughout the entire bulk of the channel as opposed to the surface (**Figure 7a**) [116].

4.7.2 Surface acoustic waves

The study of SAWs dates back to 1885, when they were initially studied by Lord Rayleigh [117]. Surface acoustic waves travel parallel to the channel surface and are thought to be confined to within ~1 wavelength of the surface. SAWs are generated

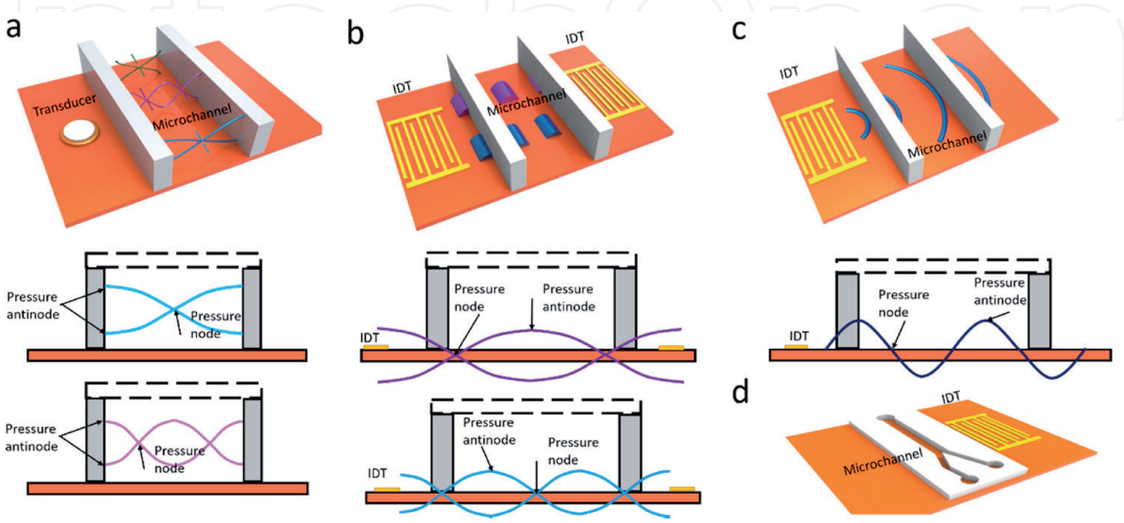

Figure 7.
Depiction of different acoustophoretic devices and concepts. (a) Depiction of bulk waves; (b) depiction of standing surface acoustic waves; (c) depiction of staveling surface acoustic waves; (d) illustration of an acoustic microfluidic device. IDT indicates interdigitated transistor. Reproduced with permission [113].

by a different transistor type - IDTs. SSAWs are characterized by fixed nodes and antinodes (**Figure 7b**). This occurs following the interference of two opposing traveling SAWs or a SAW that is reflected [113]. TSAWs, alternatively, are SAWs propagating in a single direction away from a single acoustic source (**Figure 7c**) [113]. Generally, SSAW approaches use two IDTs on opposite sides of a microfluidic device, while TSAW approaches use a single IDT single IDT (**Figure 7d**).

Particular cell types of interest can also be sorted in a contactless, label-free manner via deformation-assisted cell sorting using SAWs. SSAW fields can be applied across a channel by applying oscillation across interdigitated transducers at either side. Tunable IDT pitches give SAWs characteristic wavelengths, which can be determined using the simple equation (Eq. (1)). Where f is the frequency, v is the velocity, and x is the pitch of the transducer.

$$f = v / x \tag{1}$$

Surface acoustic wave platforms produce acoustic streaming when SAWs interact with surrounding fluid. In channels with SAW fields on either side (SSAW), hydrodynamic focusing occurs, which can also be referred to as "acoustic tweezers" [118]. The resulting streams move encapsulated cells or other particles, which can subsequently be trapped at pressure nodes along the chip [119]. For example, Nawaz et al. recently combined a real-time fluorescence and deformability cytometry method using SSAW-based label-free sorting [120]. This technique was found to reduce cell-processing time by an order of magnitude when compared to "state-of-the-art image-based sorters", without the need for specialized equipment.

Traveling surface acoustic waves (TSAWs) are less commonly used than standing surface acoustic waves (SSAWs), but have nevertheless been applied to facilitate cell sorting. Systems using traveling waves often employ them for the purpose of cellular deflection into collection wells or outlets via fluid streaming initiated by acoustic radiation forces [121, 122]. Cells can be sorted by size directly from bulk solution using a TSAWs, governed by a simple equation, adapted from the work of Skowronek et al., which describes whether acoustic radiation drag force from streaming dominates (Eq. (2)) [123]. In which K is a dimensionless factor, r is particle radius, and λ is the wavelength of the TSAW. When $K < 1$, streaming induced drag dominates. When $K > 1$, radiation dominates.

$$K = 2\pi r / \lambda \tag{2}$$

4.8 Other techniques

Certain other techniques can be used for cell sorting and manipulation that do not fit precisely into any of the aforementioned categories. An example that has garnered attention in recent years is bubble-based deflection, which can be produced by numerous different physical mechanisms.

4.8.1 Bubble-based deflection

Bubble deflection, also referred to by other terms such as 'vortex-actuated cell sorting' is a technique which uses instantaneous bubble-formation to in turn induce formation of a fluid jet which is then able to deflect cells of interest as they pass through a microchannel. Bubbles can be generated in one of several ways. The most

common approach is to use heating to induce bubble formation. For example, resistive or electrical heating can be used to heat the surrounding area [124, 125]. Heating can also be created by optical mechanisms, such as laser light pulses [62, 125–128]. Ultrasound can also be used to create bubbles via cavitation, but, due to the large wavelengths, precise sorting of cells is difficult [129]. Spark discharge in dielectric liquids causes "electrical breakdown" and can induce the formation of highly pressurized bubbles at the electrodes [130].

Bubble deflection techniques are commonly integrated into a FACS workflow as a replacement step for electrostatic charging and charge-based deflection. In this standard workflow, optical detection with fluorophores is still needed. However, certain researchers have used bubbles as cellular tags, and measured displacement and velocity under ultrasound, representing a potentially label-free technique [131].

5. Conclusion

Sample preparation for single-cell analysis is a critical area of importance for future research in order to ensure widespread clinical translation of single-cell sequencing and other single-cell analysis techniques into cancer diagnostics and other workflows. The highlighted tissue dissociation, sample purification and cell manipulation techniques represent the current state-of-the art at the time of this writing for these processing steps.

New techniques relevant to the preparation of single cells from tissues will continue to be developed in the coming years. Additionally, researchers have begun combining several of the techniques discussed herein into new, hybrid devices, some of which have even been commercialized. For example, the CTC-iChip sorts cells using a combination of deterministic lateral displacement, inertial focusing, and magnetophoresis and was able to obtain a cell sorting rate of 10^7 cells/second and an enrichment yield of 97% rare cells [132]. While this device was notably tailored towards the liquid biopsy workflow, it's quite possible that integrated techniques targeting tissue dissociation into single cells will be forthcoming.

Comprehensive sample preparation instruments that can perform tissue dissociation, purification, and single-cell selection functions, among others are a current target of innovation, as evidenced by the work of Lombardo et al., who combined dissociation with purification via filters, enabling subsequent single-cell sequencing [15].

It is likely that machine learning and artificial intelligence will continue to be combined with the sample preparation process in order to improve the workflow. Laboratory automation techniques, such as automated liquid handling systems will also continue to increase in prominence to both heighten throughput and avoid manual processing. These methods will collectively expedite processing, making it significantly more efficient and less laborious.

The combination of high-resolution single-cell analysis techniques with high-heterogeneity tissue samples represents an ideal path forward to achieving next generation cancer diagnostics. However, for this future to become a reality, much progress and innovation is still needed within the tissue to single-cell sample preparation workflow. Developments are being made on multiple fronts, from dissociation and cellular manipulation to machine learning and computer vision. Integrated techniques are on the rise, and new devices are being commercialized. A comprehensive discussion of available technologies has been presented here with particular emphasis on translational potential within the tissue to the single-cell sequencing workflow.

6. Further reading

For more information on other steps in the sample preparation workflow, such as single-cell encapsulation and lysis, we recommend Hosic et al.'s comprehensive review [23]. To contextualize the relevance of the single-cell analysis workflow, the reader is also recommended the following papers on heterogeneity and spatial resolution in tissue as resolved through single-cell analysis [133–136]. For further information on experimental considerations for particular single-cell approaches, the reader is recommended the following resources: scDNAseq [137], scRNAseq [138–140], ATAC-seq [141], single-cell proteomics [142–144] and single-cell metabolomics [145, 146].

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References

[1] E. C. Welch, J. M. Powell, T. B. Clevinger, A. E. Fairman, and A. Shukla, "Advances in Biosensors and Diagnostic Technologies Using Nanostructures and Nanomaterials," *Adv. Funct. Mater.*, Jul. 2021, doi: 10.1002/adfm.202104126.

[2] A. Vlahiotis, B. Griffin, F. A. T. Stavros MD, and J. Margolis, "Analysis of utilization patterns and associated costs of the breast imaging and diagnostic procedures after screening mammography," *Clin. Outcomes Res.*, vol. Volume 10, Mar. 2018, doi: 10.2147/CEOR.S150260.

[3] Y. Wang and N. E. Navin, "Advances and Applications of Single-Cell Sequencing Technologies," *Mol. Cell*, vol. 58, no. 4, May 2015, doi: 10.1016/j.molcel.2015.05.005.

[4] G. POSTE, "Tissue dissociation with proteolytic enzymes *1Adsorption and activity of enzymes at the cell surface," *Exp. Cell Res.*, vol. 65, no. 2, Apr. 1971, doi: 10.1016/0014-4827(71)90014-0.

[5] J. D. Robin, W. E. Wright, Y. Zou, S. C. Cossette, M. W. Lawlor, and E. Gussoni, "Isolation and Immortalization of Patient-derived Cell Lines from Muscle Biopsy for Disease Modeling," *J. Vis. Exp.*, no. 95, Jan. 2015, doi: 10.3791/52307.

[6] E. C. Welch, H. Yu, and *A. Tripathi*, "Optimization of a Clinically Relevant Chemical-Mechanical Tissue Dissociation Workflow for Single-Cell Analysis," *Cell. Mol. Bioeng.*, Mar. 2021, doi: 10.1007/s12195-021-00667-y.

[7] S. Scheuermann, A. Schäfer, J. Langejürgen, and C. Reis, "A step towards enzyme-free tissue dissociation," *Curr. Dir. Biomed. Eng.*, vol. 5, no. 1, Sep. 2019, doi: 10.1515/cdbme-2019-0137.

[8] S. M. Hattersley, C. E. Dyer, J. Greenman, and S. J. Haswell, "Development of a microfluidic device for the maintenance and interrogation of viable tissue biopsies," *Lab Chip*, vol. 8, no. 11, 2008, doi: 10.1039/b809345h.

[9] C.-H. Lin, D.-C. Lee, H.-C. Chang, I.-M. Chiu, and C.-H. Hsu, "Single-Cell Enzyme-Free Dissociation of Neurospheres Using a Microfluidic Chip," *Anal. Chem.*, vol. 85, no. 24, Dec. 2013, doi: 10.1021/ac402724b.

[10] L. Wallman *et al.*, "Biogrid—a microfluidic device for large-scale enzyme-free dissociation of stem cell aggregates," *Lab Chip*, vol. 11, no. 19, 2011, doi: 10.1039/c1lc20316a.

[11] X. Qiu *et al.*, "Microfluidic filter device with nylon mesh membranes efficiently dissociates cell aggregates and digested tissue into single cells," *Lab Chip*, vol. 18, no. 18, 2018, doi: 10.1039/C8LC00507A.

[12] X. Qiu *et al.*, "Microfluidic channel optimization to improve hydrodynamic dissociation of cell aggregates and tissue," *Sci. Rep.*, vol. 8, no. 1, Dec. 2018, doi: 10.1038/s41598-018-20931-y.

[13] X. Qiu, J. De Jesus, M. Pennell, M. Troiani, and J. B. Haun, "Microfluidic device for mechanical dissociation of cancer cell aggregates into single cells," *Lab Chip*, vol. 15, no. 1, 2015, doi: 10.1039/C4LC01126K.

[14] X. Qiu *et al.*, "Microfluidic device for rapid digestion of tissues into cellular suspensions," *Lab Chip*, vol. 17, no. 19, 2017, doi: 10.1039/C7LC00575J.

[15] J. A. Lombardo, M. Aliaghaei, Q. H. Nguyen, K. Kessenbrock, and J. B. Haun, "Microfluidic platform accelerates tissue processing into single cells for molecular analysis and primary culture models," *Nat. Commun.*, vol. 12,

no. 1, Dec. 2021, doi: 10.1038/s41467-021-23238-1.

[16] E. C. Welch and A. Tripathi, "ELECTRICAL DISSOCIATION OF TISSUE SAMPLES INTO SINGLE CELLS AND/OR SMALLER GROUPS OF CELLS," 63/177,211, 2021.

[17] G. H. Markx, "The use of electric fields in tissue engineering," *Organogenesis*, vol. 4, no. 1, Jan. 2008, doi: 10.4161/org.5799.

[18] F. X. Hart and J. R. Palisano, "The Application of Electric Fields in Biology and Medicine," in *Electric Field*, InTech, 2018.

[19] J. P. Freyer and R. M. Sutherland, "Selective Dissociation and Characterization of Cells from Different Regions of Multicell Tumor Spheroids," *Cancer Res.*, vol. 40, no. 11, pp. 3956-3965, 1980.

[20] J. P. Freyer and P. L. Schor, "Automated Selective Dissociation of Cells From Different Regions of Multicellular Spheroids," *Vitr. Cell. Dev. Biol.*, vol. 25, no. 1, pp. 9-19, 1988.

[21] M. R. Emmert-Buck *et al.*, "Laser Capture Microdissection," *Science (80-.).*, vol. 274, no. 5289, Nov. 1996, doi: 10.1126/science.274.5289.998.

[22] R. F. Bonner, "CELL SAMPLING: Laser Capture Microdissection: Molecular Analysis of Tissue," *Science (80-.).*, vol. 278, no. 5342, Nov. 1997, doi: 10.1126/science.278.5342.1481.

[23] S. Hosic, S. K. Murthy, and A. N. Koppes, "Microfluidic Sample Preparation for Single Cell Analysis," *Anal. Chem.*, vol. 88, no. 1, Jan. 2016, doi: 10.1021/acs.analchem.5b04077.

[24] L. Moulédous, S. Hunt, R. Harcourt, J. Harry, K. L. Williams, and H. B. Gutstein, "Navigated laser capture microdissection as an alternative to direct histological staining for proteomic analysis of brain samples," *Proteomics*, vol. 3, no. 5, May 2003, doi: 10.1002/pmic.200300398.

[25] B. J. Erickson, P. Korfiatis, Z. Akkus, and T. L. Kline, "Machine Learning for Medical Imaging," *RadioGraphics*, vol. 37, no. 2, Mar. 2017, doi: 10.1148/rg.2017160130.

[26] B. Karaçali and A. Tözeren, "Automated detection of regions of interest for tissue microarray experiments: an image texture analysis," *BMC Med. Imaging*, vol. 7, no. 1, Dec. 2007, doi: 10.1186/1471-2342-7-2.

[27] J. Hipp *et al.*, "Computer-aided laser dissection: A microdissection workflow leveraging image analysis tools," *J. Pathol. Inform.*, vol. 9, no. 1, 2018, doi: 10.4103/jpi.jpi_60_18.

[28] S. Ren, Y. Luo, T. Yan, L. Wang, D. Chen, and X. Chen, "Machine learning-based automatic segmentation of region of interest in dynamic optical imaging," *AIP Adv.*, vol. 11, no. 1, Jan. 2021, doi: 10.1063/5.0033605.

[29] B. Maiorella, G. Dorin, A. Carion, and D. Harano, "Crossflow microfiltration of animal cells," *Biotechnol. Bioeng.*, vol. 37, no. 2, Jan. 1991, doi: 10.1002/bit.260370205.

[30] P. Preira, V. Grandné, J.-M. Forel, S. Gabriele, M. Camara, and O. Theodoly, "Passive circulating cell sorting by deformability using a microfluidic gradual filter," *Lab Chip*, vol. 13, no. 1, 2013, doi: 10.1039/C2LC40847C.

[31] L. A. Herzenberg, R. G. Sweet, and L. A. Herzenberg, "Fluorescence-activated cell sorting," *Sci. Am.*, vol. 234, no. 3, pp. 108-118, 1976.

[32] L. A. Herzenberg, D. Parks, B. Sahaf, O. Perez, M. Roederer, and L. A. Herzenberg, "The History and Future of the Fluorescence Activated Cell Sorter

and Flow Cytometry: A View from Stanford," *Clin. Chem.*, vol. 48, no. 10, Oct. 2002, doi: 10.1093/clinchem/48.10.1819.

[33] J. Krüger, K. Singh, A. O'Neill, C. Jackson, A. Morrison, and P. O'Brien, "Development of a microfluidic device for fluorescence activated cell sorting," *J. Micromechanics Microengineering*, vol. 12, no. 4, Jul. 2002, doi: 10.1088/0960-1317/12/4/324.

[34] B. Yao, G. Luo, X. Feng, W. Wang, L. Chen, and Y. Wang, "A microfluidic device based on gravity and electric force driving for flow cytometry and fluorescence activated cell sorting," *Lab Chip*, vol. 4, no. 6, 2004, doi: 10.1039/b408422e.

[35] J. Autebert *et al.*, "Microfluidic: An innovative tool for efficient cell sorting," *Methods*, vol. 57, no. 3, Jul. 2012, doi: 10.1016/j.ymeth.2012.07.002.

[36] J.-C. Baret *et al.*, "Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity," *Lab Chip*, vol. 9, no. 13, 2009, doi: 10.1039/b902504a.

[37] H. M. Shapiro, *Practical flow cytometry.* John Wiley & Sons, 2005.

[38] O. Caen *et al.*, "High-throughput multiplexed fluorescence-activated droplet sorting," *Microsystems Nanoeng.*, vol. 4, no. 1, Dec. 2018, doi: 10.1038/s41378-018-0033-2.

[39] I. Pirozzi, A. Snider, M. Kraus, E. R. Schönbrunner, and *A. Tripathi*, "Microfluidic Immiscible Phase Filtration System for the Isolation of Small Numbers of Cells from Whole Blood," *Cytom. Part A*, vol. 95, no. 8, Aug. 2019, doi: 10.1002/cyto.a.23736.

[40] S. Miltenyi, W. Müller, W. Weichel, and A. Radbruch, "High gradient magnetic cell separation with MACS," *Cytometry*, vol. 11, no. 2, 1990, doi: 10.1002/cyto.990110203.

[41] V. Martin, Siewert C, Scharl A, and Harms T, "Immunomagnetic enrichment of disseminated epithelial tumor cells from peripheral blood by MACS."

[42] A. Palmon, R. David, Y. Neumann, R. Stiubea-Cohen, G. Krief, and D. J. Aframian, "High-efficiency immunomagnetic isolation of solid tissue-originated integrin-expressing adult stem cells," *Methods*, vol. 56, no. 2, Feb. 2012, doi: 10.1016/j.ymeth.2011.10.002.

[43] N. HARBECK *et al.*, "MODEL SYSTEM FOR ISOLATION OF COMPETENT OVARIAN-CARCINOMA CELLS FROM FRESH TUMOR-TISSUE BY A MAGNETIC SEPARATION TECHNIQUE (MACS)," *Int. J. Oncol.*, Jun. 1995, doi: 10.3892/ijo.6.6.1249.

[44] Y. Wang *et al.*, "A microfluidic robot for rare cell sorting based on machine vision identification and multi-step sorting strategy," *Talanta*, vol. 226, May 2021, doi: 10.1016/j.talanta.2021.122136.

[45] A. Ashkin, J. M. Dziedzic, J. E. Bjorkholm, and S. Chu, "Observation of a single-beam gradient force optical trap for dielectric particles," *Opt. Lett.*, vol. 11, no. 5, May 1986, doi: 10.1364/OL.11.000288.

[46] X. Wang *et al.*, "Enhanced cell sorting and manipulation with combined optical tweezer and microfluidic chip technologies," *Lab Chip*, vol. 11, no. 21, 2011, doi: 10.1039/c1lc20653b.

[47] Y. Z. Yoon and P. Cicuta, "Optical trapping of colloidal particles and cells by focused evanescent fields using conical lenses," *Opt. Express*, vol. 18, no. 7, Mar. 2010, doi: 10.1364/OE.18.007076.

[48] Y. Li, Z. Guo, and S. Qu, "Living cell manipulation in a microfluidic device by femtosecond optical tweezers," *Opt. Lasers Eng.*, vol. 55, Apr. 2014, doi: 10.1016/j.optlaseng.2013.11.001.

[49] T. Yamazaki *et al.*, "Construction of 3D Cellular Composites with Stem Cells Derived from Adipose Tissue and Endothelial Cells by Use of Optical Tweezers in a Natural Polymer Solution," *Materials (Basel).*, vol. 12, no. 11, May 2019, doi: 10.3390/ma12111759.

[50] J. P. K. Armstrong and M. M. Stevens, "Using Remote Fields for Complex Tissue Engineering," *Trends Biotechnol.*, vol. 38, no. 3, Mar. 2020, doi: 10.1016/j.tibtech.2019.07.005.

[51] X. Li, C. Liu, S. Chen, Y. Wang, S. H. Cheng, and D. Sun, "<italic>In Vivo</italic> Manipulation of Single Biological Cells With an Optical Tweezers-Based Manipulator and a Disturbance Compensation Controller," *IEEE Trans. Robot.*, vol. 33, no. 5, Oct. 2017, doi: 10.1109/TRO.2017.2718554.

[52] X. Li, H. Yang, H. Huang, and D. Sun, "A switching controller for high speed cell transportation by using a robot-aided optical tweezers system," *Automatica*, vol. 89, Mar. 2018, doi: 10.1016/j.automatica.2017.11.014.

[53] M. Xie, A. Shakoor, and C. Wu, "Manipulation of Biological Cells Using a Robot-Aided Optical Tweezers System," *Micromachines*, vol. 9, no. 5, May 2018, doi: 10.3390/mi9050245.

[54] M. C. Wu, "Optoelectronic tweezers," *Nat. Photonics*, vol. 5, no. 6, Jun. 2011, doi: 10.1038/nphoton.2011.98.

[55] A. T. Ohta *et al.*, "Dynamic Cell and Microparticle Control via Optoelectronic Tweezers," *J. Microelectromechanical Syst.*, vol. 16, no. 3, Jun. 2007, doi: 10.1109/JMEMS.2007.896717.

[56] P. Y. Chiou, A. T. Ohta, and M. C. Wu, "Massively parallel manipulation of single cells and microparticles using optical images," *Nature*, vol. 436, no. 7049, Jul. 2005, doi: 10.1038/nature03831.

[57] A. T. Ohta *et al.*, "Optically Controlled Cell Discrimination and Trapping Using Optoelectronic Tweezers," *IEEE J. Sel. Top. Quantum Electron.*, vol. 13, no. 2, 2007, doi: 10.1109/JSTQE.2007.893558.

[58] L.-Y. Ke *et al.*, "Cancer immunotherapy μ-environment LabChip: taking advantage of optoelectronic tweezers," *Lab Chip*, vol. 18, no. 1, 2018, doi: 10.1039/C7LC00963A.

[59] S. Zhang *et al.*, "The optoelectronic microrobot: A versatile toolbox for micromanipulation," *Proc. Natl. Acad. Sci.*, vol. 116, no. 30, Jul. 2019, doi: 10.1073/pnas.1903406116.

[60] K. Ramser and D. Hanstorp, "Optical manipulation for single-cell studies," *J. Biophotonics*, vol. 3, no. 4, Apr. 2010, doi: 10.1002/jbio.200910050.

[61] S.-K. Hoi, C. Udalagama, C.-H. Sow, F. Watt, and A. A. Bettiol, "Microfluidic sorting system based on optical force switching," *Appl. Phys. B*, vol. 97, no. 4, Dec. 2009, doi: 10.1007/s00340-009-3687-5.

[62] T.-H. Wu *et al.*, "Pulsed laser triggered high speed microfluidic fluorescence activated cell sorter," *Lab Chip*, vol. 12, no. 7, 2012, doi: 10.1039/c2lc21084c.

[63] C. M. Bailey-Hytholt, S. Sayeed, M. Kraus, R. Joseph, A. Shukla, and *A. Tripathi*, "A Rapid Method for Label-Free Enrichment of Rare Trophoblast Cells from Cervical Samples," *Sci. Rep.*, vol. 9, no. 1, Dec. 2019, doi: 10.1038/s41598-019-48346-3.

[64] C. M. Bailey-Hytholt, S. Sayeed, A. Shukla, and *A. Tripathi*, "Enrichment of Placental Trophoblast Cells from Clinical Cervical Samples Using Differences in Surface Adhesion on an Inclined Plane," *Ann. Biomed. Eng.*, Mar. 2021, doi: 10.1007/s10439-021-02742-x.

[65] J. D. Amack and M. L. Manning, "Knowing the Boundaries: Extending the Differential Adhesion Hypothesis in Embryonic Cell Sorting," *Science (80-.).*, vol. 338, no. 6104, Oct. 2012, doi: 10.1126/science.1223953.

[66] S. L. Stott *et al.*, "Isolation of circulating tumor cells using a microvortex-generating herringbone-chip," *Proc. Natl. Acad. Sci.*, vol. 107, no. 43, Oct. 2010, doi: 10.1073/pnas.1012539107.

[67] Y. Chikaishi, K. Yoneda, T. Ohnaga, and F. Tanaka, "EpCAM-independent capture of circulating tumor cells with a 'universal CTC-chip,'" *Oncol. Rep.*, vol. 37, no. 1, Jan. 2017, doi: 10.3892/or.2016.5235.

[68] K. Pu, C. Li, N. Zhang, H. Wang, W. Shen, and Y. Zhu, "Epithelial cell adhesion molecule independent capture of non-small lung carcinoma cells with peptide modified microfluidic chip," *Biosens. Bioelectron.*, vol. 89, Mar. 2017, doi: 10.1016/j.bios.2016.09.092.

[69] L. R. Huang, "Continuous Particle Separation Through Deterministic Lateral Displacement," *Science (80-.).*, vol. 304, no. 5673, May 2004, doi: 10.1126/science.1094567.

[70] J. McGrath, M. Jimenez, and H. Bridle, "Deterministic lateral displacement for particle separation: a review," *Lab Chip*, vol. 14, no. 21, 2014, doi: 10.1039/C4LC00939H.

[71] A. Hochstetter *et al.*, "Deterministic Lateral Displacement: Challenges and Perspectives," *ACS Nano*, vol. 14, no. 9, Sep. 2020, doi: 10.1021/acsnano.0c05186.

[72] J. V. Green, M. Radisic, and S. K. Murthy, "Deterministic Lateral Displacement as a Means to Enrich Large Cells for Tissue Engineering," *Anal. Chem.*, vol. 81, no. 21, Nov. 2009, doi: 10.1021/ac9018395.

[73] Z. Liu *et al.*, "Rapid isolation of cancer cells using microfluidic deterministic lateral displacement structure," *Biomicrofluidics*, vol. 7, no. 1, Jan. 2013, doi: 10.1063/1.4774308.

[74] K. Loutherback, K. S. Chou, J. Newman, J. Puchalla, R. H. Austin, and J. C. Sturm, "Improved performance of deterministic lateral displacement arrays with triangular posts," *Microfluid. Nanofluidics*, vol. 9, no. 6, Dec. 2010, doi: 10.1007/s10404-010-0635-y.

[75] S. Choi, J. M. Karp, and R. Karnik, "Cell sorting by deterministic cell rolling," *Lab Chip*, vol. 12, no. 8, 2012, doi: 10.1039/c2lc21225k.

[76] D. Di Carlo, D. Irimia, R. G. Tompkins, and M. Toner, "Continuous inertial focusing, ordering, and separation of particles in microchannels," *Proc. Natl. Acad. Sci.*, vol. 104, no. 48, Nov. 2007, doi: 10.1073/pnas.0704958104.

[77] J. M. Martel and M. Toner, "Inertial focusing dynamics in spiral microchannels," *Phys. Fluids*, vol. 24, no. 3, Mar. 2012, doi: 10.1063/1.3681228.

[78] A. Al-Halhouli *et al.*, "Enhanced inertial focusing of microparticles and cells by integrating trapezoidal microchambers in spiral microfluidic channels," *RSC Adv.*, vol. 9, no. 33, 2019, doi: 10.1039/C9RA03587G.

[79] A. Shiriny and M. Bayareh, "Inertial focusing of CTCs in a novel spiral microchannel," *Chem. Eng. Sci.*, vol. 229, Jan. 2021, doi: 10.1016/j.ces.2020.116102.

[80] Y. Gou *et al.*, "Sheathless Inertial Focusing Chip Combining a Spiral Channel with Periodic Expansion Structures for Efficient and Stable Particle Sorting," *Anal. Chem.*, vol. 92, no. 2, Jan. 2020, doi: 10.1021/acs.analchem.9b03692.

[81] H. Ren *et al.*, "Multiplexed serpentine microchannels for

high-throughput sorting of disseminated tumor cells from malignant pleural effusion," *Sensors Actuators B Chem.*, vol. 337, Jun. 2021, doi: 10.1016/j.snb.2021.129758.

[82] D. Tang, M. Chen, Y. Han, N. Xiang, and Z. Ni, "Asymmetric serpentine microchannel based impedance cytometer enabling consistent transit and accurate characterization of tumor cells and blood cells," *Sensors Actuators B Chem.*, vol. 336, Jun. 2021, doi: 10.1016/j.snb.2021.129719.

[83] V. Choesmel *et al.*, "Enrichment methods to detect bone marrow micrometastases in breast carcinoma patients: clinical relevance," *Breast Cancer Res.*, vol. 6, no. 5, Oct. 2004, doi: 10.1186/bcr898.

[84] R. Gorkin *et al.*, "Centrifugal microfluidics for biomedical applications," *Lab Chip*, vol. 10, no. 14, 2010, doi: 10.1039/b924109d.

[85] A. Snider, I. Pirozzi, and *A. Tripathi*, "Centrifugal Microfluidics Traps for Parallel Isolation and Imaging of Single Cells," *Micromachines*, vol. 11, no. 2, Jan. 2020, doi: 10.3390/mi11020149.

[86] O. Strohmeier *et al.*, "Centrifugal microfluidic platforms: advanced unit operations and applications," *Chem. Soc. Rev.*, vol. 44, no. 17, 2015, doi: 10.1039/C4CS00371C.

[87] X. Zhang, Y. Li, S. Shen, S. Lee, and H. Dou, "Field-flow fractionation: A gentle separation and characterization technique in biomedicine," *TrAC Trends Anal. Chem.*, vol. 108, Nov. 2018, doi: 10.1016/j.trac.2018.09.005.

[88] C. Mélin *et al.*, "Cancer Stem Cell Sorting from Colorectal Cancer Cell Lines by Sedimentation Field Flow Fractionation," *Anal. Chem.*, vol. 84, no. 3, Feb. 2012, doi: 10.1021/ac202797z.

[89] S. Wang *et al.*, "Microalgae separation by inertia-enhanced pinched flow fractionation," *Electrophoresis*, May 2021, doi: 10.1002/elps.202000325.

[90] J. T. W. Berendsen, J. C. T. Eijkel, A. M. Wetzels, and L. I. Segerink, "Separation of spermatozoa from erythrocytes using their tumbling mechanism in a pinch flow fractionation device," *Microsystems Nanoeng.*, vol. 5, no. 1, Dec. 2019, doi: 10.1038/s41378-019-0068-z.

[91] X.-B. Wang, J. Yang, Y. Huang, J. Vykoukal, F. F. Becker, and P. R. C. Gascoyne, "Cell Separation by Dielectrophoretic Field-flow-fractionation," *Anal. Chem.*, vol. 72, no. 4, Feb. 2000, doi: 10.1021/ac990922o.

[92] A. Shamloo and A. Kamali, "Numerical analysis of a dielectrophoresis field-flow fractionation device for the separation of multiple cell types," *J. Sep. Sci.*, vol. 40, no. 20, Oct. 2017, doi: 10.1002/jssc.201700325.

[93] C. F. Gonzalez and V. T. Remcho, "Harnessing dielectric forces for separations of cells, fine particles and macromolecules," *J. Chromatogr. A*, vol. 1079, no. 1-2, Jun. 2005, doi: 10.1016/j.chroma.2005.03.070.

[94] C. Wyatt Shields IV, C. D. Reyes, and G. P. López, "Microfluidic cell sorting: a review of the advances in the separation of cells from debulking to rare cell isolation," *Lab Chip*, vol. 15, no. 5, 2015, doi: 10.1039/C4LC01246A.

[95] B. Yan, B. Chen, F. Liu, J. Wu, and Y. Xiong, "Combining field-modulating electroosmotic vortex and insulating post to manipulate particles based on dielectrophoresis," *Appl. Math. Mech.*, vol. 42, no. 3, Mar. 2021, doi: 10.1007/s10483-021-2706-5.

[96] L. Li, X. Wang, Q. Pu, and S. Liu, "Advancement of electroosmotic pump

in microflow analysis: A review," *Anal. Chim. Acta*, vol. 1060, Jul. 2019, doi: 10.1016/j.aca.2019.02.004.

[97] M. Alipanah, M. Hafttananian, N. Hedayati, A. Ramiar, and M. Alipanah, "Microfluidic on-demand particle separation using induced charged electroosmotic flow and magnetic field," *J. Magn. Magn. Mater.*, vol. 537, Nov. 2021, doi: 10.1016/j.jmmm.2021.168156.

[98] S. Valagerahally Puttaswamy, S. Sivashankar, C.-H. Yeh, R.-J. Chen, and C. H. Liu, "Electrodynamically actuated on-chip flow cytometry with low shear stress for electro-osmosis based sorting using low conductive medium," *Microelectron. Eng.*, vol. 87, no. 12, Dec. 2010, doi: 10.1016/j.mee.2010.07.023.

[99] X. Ou, *P. Chen*, X. Huang, S. Li, and B. Liu, "Microfluidic chip electrophoresis for biochemical analysis," *J. Sep. Sci.*, vol. 43, no. 1, Jan. 2020, doi: 10.1002/jssc.201900758.

[100] W. Zhang, N. Li, H. Zeng, H. Nakajima, J.-M. Lin, and K. Uchiyama, "Inkjet Printing Based Separation of Mammalian Cells by Capillary Electrophoresis," *Anal. Chem.*, vol. 89, no. 17, Sep. 2017, doi: 10.1021/acs.analchem.7b02624.

[101] F. Guo *et al.*, "Droplet electric separator microfluidic device for cell sorting," *Appl. Phys. Lett.*, vol. 96, no. 19, May 2010, doi: 10.1063/1.3360812.

[102] J. Yoshioka, Y. Ohsugi, T. Yoshitomi, T. Yasukawa, N. Sasaki, and K. Yoshimoto, "Label-Free Rapid Separation and Enrichment of Bone Marrow-Derived Mesenchymal Stem Cells from a Heterogeneous Cell Mixture Using a Dielectrophoresis Device," *Sensors*, vol. 18, no. 9, Sep. 2018, doi: 10.3390/s18093007.

[103] M. Elitas, Y. Yildizhan, M. Islam, R. Martinez-Duarte, and D. Ozkazanc, "Dielectrophoretic characterization and separation of monocytes and macrophages using 3D carbon-electrodes," *Electrophoresis*, vol. 40, no. 2, Jan. 2019, doi: 10.1002/elps.201800324.

[104] K. Kikkeri, B. A. Kerr, A. S. Bertke, J. S. Strobl, and M. Agah, "Passivated-electrode insulator-based dielectrophoretic separation of heterogeneous cell mixtures," *J. Sep. Sci.*, vol. 43, no. 8, Apr. 2020, doi: 10.1002/jssc.201900553.

[105] Y.-C. Kung, K. R. Niazi, and P.-Y. Chiou, "Tunnel dielectrophoresis for ultra-high precision size-based cell separation," *Lab Chip*, vol. 21, no. 6, 2021, doi: 10.1039/D0LC00853B.

[106] A. Sonnenberg *et al.*, "Dielectrophoretic isolation and detection of cfc-DNA nanoparticulate biomarkers and virus from blood," *Electrophoresis*, vol. 34, no. 7, Apr. 2013, doi: 10.1002/elps.201200444.

[107] F. S. O. Fritzsch *et al.*, "Picoliter nDEP traps enable time-resolved contactless single bacterial cell analysis in controlled microenvironments," *Lab Chip*, vol. 13, no. 3, 2013, doi: 10.1039/C2LC41092C.

[108] S. Bhattacharya *et al.*, "Selective trapping of single mammalian breast cancer cells by insulator-based dielectrophoresis," *Anal. Bioanal. Chem.*, vol. 406, no. 7, Mar. 2014, doi: 10.1007/s00216-013-7598-2.

[109] L. Wang, L. A. Flanagan, N. L. Jeon, E. Monuki, and A. P. Lee, "Dielectrophoresis switching with vertical sidewall electrodes for microfluidic flow cytometry," *Lab Chip*, vol. 7, no. 9, 2007, doi: 10.1039/b705386j.

[110] Y. Wu, Y. Ren, Y. Tao, and H. Jiang, "Fluid pumping and cells separation by DC-biased traveling wave electroosmosis and dielectrophoresis," *Microfluid. Nanofluidics*, vol. 21, no. 3,

Mar. 2017, doi: 10.1007/s10404-017-1862-2.

[111] M. Yang and X. Zhang, "Electrical assisted patterning of cardiac myocytes with controlled macroscopic anisotropy using a microfluidic dielectrophoresis chip," *Sensors Actuators A Phys.*, vol. 135, no. 1, Mar. 2007, doi: 10.1016/j.sna.2006.06.071.

[112] W. M. Arnold and U. Zimmermann, "Electro-rotation: development of a technique for dielectric measurements on individual cells and particles," *J. Electrostat.*, vol. 21, no. 2-3, Sep. 1988, doi: 10.1016/0304-3886(88)90027-7.

[113] Y. Gao, M. Wu, Y. Lin, and J. Xu, "Acoustic Microfluidic Separation Techniques and Bioapplications: A Review," *Micromachines*, vol. 11, no. 10, Oct. 2020, doi: 10.3390/mi11100921.

[114] L. Johansson, F. Nikolajeff, S. Johansson, and S. Thorslund, "On-Chip Fluorescence-Activated Cell Sorting by an Integrated Miniaturized Ultrasonic Transducer," *Anal. Chem.*, vol. 81, no. 13, Jul. 2009, doi: 10.1021/ac802681r.

[115] I. Leibacher, P. Reichert, and J. Dual, "Microfluidic droplet handling by bulk acoustic wave (BAW) acoustophoresis," *Lab Chip*, vol. 15, no. 13, 2015, doi: 10.1039/C5LC00083A.

[116] G. P. Gautam, T. Burger, A. Wilcox, M. J. Cumbo, S. W. Graves, and M. E. Piyasena, "Simple and inexpensive micromachined aluminum microfluidic devices for acoustic focusing of particles and cells," *Anal. Bioanal. Chem.*, vol. 410, no. 14, May 2018, doi: 10.1007/s00216-018-1034-6.

[117] Lord Rayleigh, "On Waves Propagated along the Plane Surface of an Elastic Solid," *Proc. London Math. Soc.*, vol. s1-17, no. 1, Nov. 1885, doi: 10.1112/plms/s1-17.1.4.

[118] X. Ding *et al.*, "On-chip manipulation of single microparticles, cells, and organisms using surface acoustic waves," *Proc. Natl. Acad. Sci.*, vol. 109, no. 28, Jul. 2012, doi: 10.1073/pnas.1209288109.

[119] S.-C. S. Lin, X. Mao, and T. J. Huang, "Surface acoustic wave (SAW) acoustophoresis: now and beyond," *Lab Chip*, vol. 12, no. 16, 2012, doi: 10.1039/c2lc90076a.

[120] A. A. Nawaz *et al.*, "Intelligent image-based deformation-assisted cell sorting with molecular specificity," *Nat. Methods*, vol. 17, no. 6, Jun. 2020, doi: 10.1038/s41592-020-0831-y.

[121] L. Schmid, D. A. Weitz, and T. Franke, "Sorting drops and cells with acoustics: acoustic microfluidic fluorescence-activated cell sorter," *Lab Chip*, vol. 14, no. 19, 2014, doi: 10.1039/C4LC00588K.

[122] G. Destgeer, B. H. Ha, J. Park, J. H. Jung, A. Alazzam, and H. J. Sung, "Travelling Surface Acoustic Waves Microfluidics," *Phys. Procedia*, vol. 70, 2015, doi: 10.1016/j.phpro.2015.08.028.

[123] V. Skowronek, R. W. Rambach, L. Schmid, K. Haase, and T. Franke, "Particle Deflection in a Poly(dimethylsiloxane) Microchannel Using a Propagating Surface Acoustic Wave: Size and Frequency Dependence," *Anal. Chem.*, vol. 85, no. 20, Oct. 2013, doi: 10.1021/ac402607p.

[124] K. de Wijs *et al.*, "Micro vapor bubble jet flow for safe and high-rate fluorescence-activated cell sorting," *Lab Chip*, vol. 17, no. 7, 2017, doi: 10.1039/C6LC01560C.

[125] R. H. Pritchard *et al.*, "Cell sorting actuated by a microfluidic inertial vortex," *Lab Chip*, vol. 19, no. 14, 2019, doi: 10.1039/C9LC00120D.

[126] B. Han, K. Köhler, K. Jungnickel, R. Mettin, W. Lauterborn, and A. Vogel,

"Dynamics of laser-induced bubble pairs," *J. Fluid Mech.*, vol. 771, May 2015, doi: 10.1017/jfm.2015.183.

[127] Y. Chen, T.-H. Wu, Y.-C. Kung, M. A. Teitell, and P.-Y. Chiou, "3D pulsed laser-triggered high-speed microfluidic fluorescence-activated cell sorter," *Analyst*, vol. 138, no. 24, 2013, doi: 10.1039/c3an01266b.

[128] Y. Chen, A. J. Chung, T.-H. Wu, M. A. Teitell, D. Di Carlo, and P.-Y. Chiou, "Pulsed Laser Activated Cell Sorting with Three Dimensional Sheathless Inertial Focusing," *Small*, vol. 10, no. 9, May 2014, doi: 10.1002/smll.201302885.

[129] C. Lee *et al.*, "Microfluidic droplet sorting with a high frequency ultrasound beam," *Lab Chip*, vol. 12, no. 15, 2012, doi: 10.1039/c2lc21123h.

[130] J. Zhao and Z. You, "Spark-generated microbubble cell sorter for microfluidic flow cytometry," *Cytom. Part A*, vol. 93, no. 2, Feb. 2018, doi: 10.1002/cyto.a.23296.

[131] T. J. Matula *et al.*, "Ultrasound-based cell sorting with microbubbles: A feasibility study," *J. Acoust. Soc. Am.*, vol. 144, no. 1, Jul. 2018, doi: 10.1121/1.5044405.

[132] N. M. Karabacak *et al.*, "Microfluidic, marker-free isolation of circulating tumor cells from blood samples," *Nat. Protoc.*, vol. 9, no. 3, Mar. 2014, doi: 10.1038/nprot.2014.044.

[133] Q. F. Wills *et al.*, "Single-cell gene expression analysis reveals genetic associations masked in whole-tissue experiments," *Nat. Biotechnol.*, vol. 31, no. 8, Aug. 2013, doi: 10.1038/nbt.2642.

[134] M. Schelker *et al.*, "Estimation of immune cell content in tumour tissue using single-cell RNA-seq data," *Nat. Commun.*, vol. 8, no. 1, Dec. 2017, doi: 10.1038/s41467-017-02289-3.

[135] X. Wang *et al.*, "Three-dimensional intact-tissue sequencing of single-cell transcriptional states," *Science (80-.).*, vol. 361, no. 6400, Jul. 2018, doi: 10.1126/science.aat5691.

[136] K. Achim *et al.*, "High-throughput spatial mapping of single-cell RNA-seq data to tissue of origin," *Nat. Biotechnol.*, vol. 33, no. 5, May 2015, doi: 10.1038/nbt.3209.

[137] H. Zahn *et al.*, "Scalable whole-genome single-cell library preparation without preamplification," *Nat. Methods*, vol. 14, no. 2, Feb. 2017, doi: 10.1038/nmeth.4140.

[138] Q. H. Nguyen, N. Pervolarakis, K. Nee, and K. Kessenbrock, "Experimental Considerations for Single-Cell RNA Sequencing Approaches," *Front. Cell Dev. Biol.*, vol. 6, Sep. 2018, doi: 10.3389/fcell.2018.00108.

[139] A. Lafzi, C. Moutinho, S. Picelli, and H. Heyn, "Tutorial: guidelines for the experimental design of single-cell RNA sequencing studies," *Nat. Protoc.*, vol. 13, no. 12, Dec. 2018, doi: 10.1038/s41596-018-0073-y.

[140] Y. Bykov, S. H. Kim, and D. Zamarin, "Preparation of single cells from tumors for single-cell RNA sequencing," 2020.

[141] M. R. Corces *et al.*, "An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues," *Nat. Methods*, vol. 14, no. 10, Oct. 2017, doi: 10.1038/nmeth.4396.

[142] W. Liu *et al.*, "Sample preparation method for isolation of single-cell types from mouse liver for proteomic studies," *Proteomics*, vol. 11, no. 17, Sep. 2011, doi: 10.1002/pmic.201100157.

[143] M. Dou *et al.*, "High-Throughput Single Cell Proteomics Enabled by Multiplex Isobaric Labeling in a

Nanodroplet Sample Preparation Platform," *Anal. Chem.*, vol. 91, no. 20, Oct. 2019, doi: 10.1021/acs.analchem.9b03349.

[144] S. M. Williams *et al.*, "Automated Coupling of Nanodroplet Sample Preparation with Liquid Chromatography–Mass Spectrometry for High-Throughput Single-Cell Proteomics," *Anal. Chem.*, vol. 92, no. 15, Aug. 2020, doi: 10.1021/acs.analchem.0c01551.

[145] K. D. Duncan, J. Fyrestam, and I. Lanekoff, "Advances in mass spectrometry based single-cell metabolomics," *Analyst*, vol. 144, no. 3, 2019, doi: 10.1039/C8AN01581C.

[146] A. Ali *et al.*, "Single-cell metabolomics by mass spectrometry: Advances, challenges, and future applications," *TrAC Trends Anal. Chem.*, vol. 120, Nov. 2019, doi: 10.1016/j.trac.2019.02.033.