1
Types of Clinical Samples and Cellular Enrichment Strategies

Koh Meng Aw Yong, Zeta Tak For Yu, Krystal Huijiao Guan, and Jianping Fu

1.1
Introduction

The study of cells within the native tissue or at a single-cell level falls under the broad field of cellular pathology. Rudolf Virchow, widely regarded as the father of modern pathology, espoused the principle of examining cells as a method of obtaining information on the patient's well-being [1]. Although technology has evolved to allow clinicians and researchers to adopt better ways of examining cells from the tissue level all the way to the subcellular level, the underlying principle has remained unchanged throughout the years. There are many different types of biological samples regularly handled in the clinic, and they are mostly solid or liquid in nature. It is important to note that not all clinical samples will contain cells. Examples of solid clinical samples include tissues obtained through a biopsy or surgical excision, while liquid clinical samples include blood or urine. Examining cells from such clinical samples can fall under two independent but not mutually exclusive categories: visual examination of cellular morphology under the microscope or analyzing the molecular makeup of the cell. With advances in molecular biology, it is now possible to sequence the genome and study the gene expression at the single-cell level [2]. Although such high sensitivity permits the analysis of rare single cells, it is critical that specimen preparation is clean and free of contamination to ensure specificity of analysis.

In this chapter, we first briefly discuss the types of clinical samples available, focusing primarily on the ones that contain cells (Section 1.2). In Section 1.3, we discuss about the conventional technology currently used for cell enrichment. In Section 1.4, we review some of the micro- and nanoscale microfluidic devices, their underlying principles, and how these devices are rapidly changing the ways we approach cellular enrichment from clinical samples.

Micro- and Nanosystems for Biotechnology, First Edition. Edited by J. Christopher Love.

1.2 Types of Clinical Samples

Clinical samples are mainly distinguished into two types: solid or liquid. Solid samples include pieces of tissues harvested during biopsies or surgery and can be either fresh or fixed in a fixative. Liquid samples include bodily fluids such as blood or urine. Depending on the type of downstream processing required, different additives may be added to liquid samples. This section briefly describes each category and provides information on the types of cells typically found in each category.

1.2.1 Solid Clinical Samples

In a hospital setting, solid clinical samples are obtained for the primary purposes of either obtaining a clinical diagnosis or to preserve the patient's well-being. In diseases such as cancer, a biopsy is recommended if the clinician determines the patient is at risk of having cancer. The entire biopsy is processed and examined visually under a microscope by a pathologist for the presence of cancer. Depending on the type of cancer, different methods of obtaining biopsies may be conducted. In suspected cases of melanoma, which occurs on the epidermis, a biopsy is typically harvested from the part of the skin where the suspected melanoma is situated through the use of a surgical blade [3]. In other cancers, such as prostate cancer, where the tissue is not easily accessible, needle biopsies are performed. The prostate is first located using ultrasound and a biopsy is obtained transrectally through the use of a biopsy needle and gun. Once the tissue biopsy is harvested, it is placed in fixative and sent to a clinical laboratory for further processing and staining before being examined by a pathologist under the microscope for the presence of cancer [4, 5]. There are other diseases apart from cancer, such as hepatitis, myopathies, or lupus that may require tissue biopsies [6–9]. In hepatitis, a liver biopsy is performed to determine the extent of fibrosis that has occurred in the liver [10]. In myopathy, a muscle biopsy is required to determine the degree of muscle atrophy as well as to make a clinical diagnosis on the type of myopathy the patient might be suffering from [6, 8]. In systemic lupus erythematosus (SLE), a form of autoimmune disease, biopsies can be extracted from multiple tissue types such as skin or kidney to provide better information on whether the patient is suffering from SLE and to determine the severity of the disease [7].

Larger clinical samples can be obtained through surgical resection. Such situations occur when there is a need to remove part or whole organs to preserve the health and well-being of the patient. This is most commonly performed in cancers of the prostate, breast, or colon to name a few examples and is an invaluable resource for studying tumor heterogeneity [11–13]. In prostate cancer, if cancer was detected in the biopsy, a decision may be made by the clinician to perform radical prostatectomy. During this procedure, the entire prostate is removed from the prostate and surrounding lymph nodes may be resected. The harvested prostate

and lymph nodes are placed in fixative, stained, and sent to the pathologist for grading. This is an important step as the clinicians need to know whether the surgical margins are clear, indicating full resection of the tumor or whether the cancer had already invaded out of the prostate and into surrounding tissues such as the lymph nodes [14]. Therefore, it is apparent that the purpose of removing solid tissue from a patient is to achieve both clinical diagnosis as well as removing any diseased tissue. If fresh human tissue is desired for research purposes, it often involves coordinating with a pathologist, setting up an internal review board for complying with the ethical implications of using human research subjects as well as informing and obtaining patient consent to participate in the study [15].

In the research setting, fresh tissue samples can be readily obtained from animal sources. Although animal use still requires adherence to ethical treatment of the animals, tissue samples from animals are more abundant and easily accessible as they do not involve patients' clinical diagnoses and the number of animals can be increased easily through purchase. Furthermore, animal facilities can and are often situated closer to a research laboratory, allowing for shorter tissue-processing times.

Animal models are typically used for the sake of studying the progression of normal development or developing treatment strategies against human diseases such as cancer, in a preclinical setting. In studying cancer treatments, the disease is first induced in animals, which can be done through the use of xenograft transplant of human cancer cells, the use of transgenic animals that bear a mutation that makes them susceptible to developing the cancer of interest, or through the use of carcinogens. Once the cancer is initiated, the animal may be treated with different types of drugs to test the efficacy of the drug in treating cancer. This is an important preclinical step as the efficacy and toxicity of the drug need to be demonstrated in animals before it can be possibly considered for future use in human subjects. At the end of the study, the animals are euthanized and various organs, tissues, or the tumor itself are harvested from the animal for downstream cellular enrichment and analysis [16].

1.2.1.1 Cellular Subtypes Found in Solid Clinical Samples

There are many different types of organs and tissues in the body each with different cells. Due to space constraints, we are unfortunately unable to cover everything. Instead, we provide a brief introduction to some of the different cell types typically encountered when working with solid tissue samples. The goal is to highlight the diversity of cellular subtypes found within any tissue sample. Specifically, we discuss the epithelium, a form of tissue that is highly abundant in the human body, as well as the underlying connective tissue.

The epithelium performs many functions, one of which is to act as a physical barrier between the environment and other tissue. At the same time, it is also responsible for selective transport of molecules such as oxygen (lung) into the blood, nutrients (intestine), or secretion of enzymes (salivary gland). It can exist as a single (simple epithelium) or multiple layers (stratified epithelium) of cells

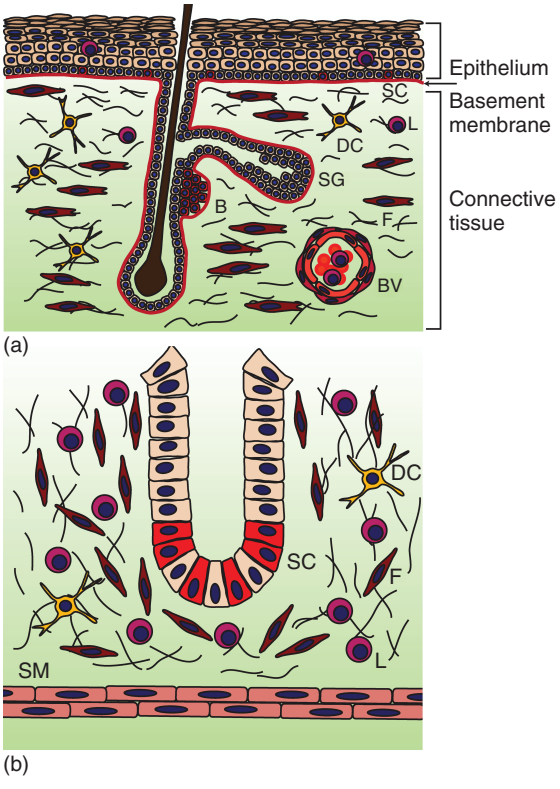

Figure 1.1 Schematic representation of stratified epithelium and underlying connective tissue (a) and simple intestinal epithelial crypt (b). Typical cells found in the epithelium and connective tissue include epithelial cells (E), dendritic cells (DC), lymphocytes (L), fibroblasts (F), and smooth muscle cells (SM). Stem cells (SC) can be found within the crypts of intestinal epithelium or within specialized compartments such as the bulge (B) in stratified epithelium. Additional structures such as blood vessels (BV) or sebaceous glands (SG) can be observed as well.

(Figure 1.1) [17]. Examples of single epithelium can be found in intestinal tissue, while stratified epithelium can be found in the epidermis. Immediately underneath the epithelium separated by a basement membrane lies the connective tissue. While the epithelium itself consists of either a single or a few layers of cells, it is often harvested together with the underlying connective tissue during biopsies or surgical resection. Therefore, most epithelial tissue samples are usually a mix of cell types of different origins (Figure 1.1). Some of the cell types observed in such samples are discussed in the remainder of this chapter. They include but are not limited to epithelial cells, fibroblasts, endothelial cells, stem cells, and immune cells. The connective tissue is also composed of a network of noncellular components, usually fibrous proteins such as collagen, proteoglycans, and glycoproteins. Growth factors and clotting factors can also be found bound within the connective tissue [18]. In diseases involving the epithelium such as cancer or pathogenic

infection, additional cell types such as cancer cells, bacteria, or parasites may be observed in the clinical samples.

As described earlier, epithelial cells serve as a barrier between the external environment and other forms of tissue. They are easily identified under the microscope due to their location, uniform appearance, and single or multilayered arrangement. They also express genes that can be used for identification on the molecular level, such as keratins, epithelial calcium-dependent adhesions (E-cadherin) and epithelial cell adhesion molecule (Ep-CAM) [19–21].

Fibroblasts are abundantly found within the connective tissue and appear morphologically different from epithelial cells. Unlike epithelial cells, fibroblasts are not arranged orderly and possess a spindle-like shape with protrusions. Although the gene signature of fibroblasts varies widely depending on the organ, it is very different from epithelial cells. Some of the common genes expressed by fibroblasts include vimentin, collagen, or smooth muscle actin [22].

The presence of small blood vessels within the connective tissue serves to transport nutrients and to remove waste from the surrounding tissue. This ensures that the epithelium, which does not possess blood vessels, is able to survive. These blood vessels are made up of endothelial cells that make up the inner lining of the vessel and are typically surrounded by smooth muscle cells that help prevent rupture of blood vessels due to constant exposure to physical forces such as stretch or pressure as blood is being pumped through. Other telltale signs of a blood vessel also include the presence of blood cells within the lumen of the vessel [17]. A further discussion on blood is available in Section 1.2.2.1.

Due to extended periods of exposure to harsh environmental conditions or lack of nutrients, epithelial cells need to be renewed regularly. Renewal of dying epithelial cells comes from the stem cell compartment found within the epithelial layer. Depending on the tissue type, stem cells may be found within different areas. In stratified epithelium such as skin, the stem cells are typically found in a region near the sebaceous gland called the *bulge* as well as within the basal cell layer (Figure 1.1) [23, 24]. In simple epithelium such as intestine, the epithelium is folded into secondary structures and forms a compartment called the *crypt*. Although it is part of the epithelium, it is believed that the stem cells actually reside within the crypt (Figure 1.1) [25].

The immune system is made up of many different cell types, and they can be found almost throughout the body in most tissue types. The immune system serves to protect the host from foreign pathogens and plays a role in regulating wound healing as well. Immune cells typically found in solid tissue include antigen-presenting cells (APCs) such as dendritic cells, macrophages, and lymphocytes. APCs constantly sample the environment for the presence of foreign pathogens. They are also involved in the processing of foreign pathogens into small peptides (antigens) as well as the presentation of antigens to lymphocytes. In doing so, APCs play a crucial role in regulating the immune system. When a foreign antigen presented by an APC is recognized by a lymphocyte, additional biochemical signals are sent to recruit and activate other immune cells [26–28].

When dealing with diseases such as cancer, the clinical sample will often contain cancer cells. Most cancers are epithelial in origin and appear morphologically different from normal epithelial cells under the microscope [29]. Among some of the key morphological differences are loss of tissue architecture, loss of cell shape, enlarged nuclei, and prominent nucleoli [30, 31]. These key morphological differences allow the pathologist to determine whether a patient has cancer and the extent to which the cancer has progressed [31, 32].

In summary, one can expect to encounter a variety of cell types when working with solid biospecimens. While these cells can be easily distinguished under the microscope using histology, enriching for a specific population of cells from this mix poses a challenge. As is discussed later in Section 1.3, we introduce several methods that can be used to enrich for specific cell populations.

1.2.2 Liquid Clinical Samples and Cellular Subtypes

Similar to solid samples, liquid clinical samples are of equal importance in clinical and research applications. Despite differences in the physical properties between solid and liquid samples, both classes contain a mix of various cell types. Examples of liquid samples include blood, urine, cerebrospinal fluid (CSF), saliva, bone marrow, and umbilical cord blood.

1.2.2.1 Blood

Blood is probably the most commonly processed form of clinical sample in the body. It is responsible for maintaining homeostasis, transporting nutrients, cells, and waste to and from tissues, making blood an extremely good indicator of overall patient health. It is also is a highly heterogeneous mix of cellular and noncellular components. For the purposes of this chapter, we only focus on the types of cells found in blood.

The most abundant cell type found in blood is the erythrocyte (also known as red blood cell, RBC), numbering about 4.1×10^9 cells ml^{-1} to 5.9×10^9 cells ml^{-1}. Mature RBCs do not have a nucleus and are mainly involved in the transport of oxygen and the removal of carbon dioxide. RBCs possess a distinct shape characterized by a biconcave disk and are easily identifiable under the microscope. Platelets are the second most abundant in blood, numbering about 1.5×10^8 counts ml^{-1} to 4.5×10^8 counts ml^{-1}. Platelets are small cellular fragments of megakaryocytes and play an important role in the clotting of blood following injury. This is crucial to ensure prevention of excessive blood loss. White blood cells (WBCs or also known as *leukocytes*) are the least common, constituting less than 1% of blood cells. However, they are major constituents of the immune system, and the numbers of WBCs in blood can range from 3.7×10^6 cells ml^{-1} to 11.1×10^6 cells ml^{-1} [33]. There are also different types of WBCs, each playing a distinct immunological role (Table 1.1). Neutrophils and monocytes are involved in the ingestion of pathogens as well as the presentation

Table 1.1 Size and abundance of cells or platelets found in blood.

Type of cell	Erythrocytes	Platelets	Neutrophils	Eosinophils	Basophils	Monocytes	Lymphocytes
Size (μm)	~7	2–3	12–15	12–17	10–14	15–20	10–16
Abundance (cells ml^{-1})	4.1–5.9 $\times 10^9$	1.5–4.5 $\times 10^8$	1.7–6.1 $\times 10^6$	0.03–0.46 $\times 10^6$	0.02–0.09 $\times 10^6$	0.2–0.6 $\times 10^6$	1.1–4.8 $\times 10^6$
Molecular markers		CD61 [34]	CD45, CD15	CD45, CD15	CD45, CD15	CD45, CD14	CD45, CD3, CD4 (T-cell), CD8 (T-cells), CD19 (B-cells)
Function	Transport and exchange of gases	Clotting	Ingestion of pathogens, regulation of immune system	Inflammation/ allergic reaction	Inflammation/ allergic reaction	Ingestion of pathogens, regulation of immune system	Cell killing, antibody production, regulation of immune response

of foreign antigens to lymphocytes and are the initiators of the immune system, while eosinophils and basophils are involved in allergic and inflammatory responses. Lymphocytes are a mix of different cell types, mainly B cells and T cells. Lymphocytes are involved in the adaptive immunity and play an active role in the killing of foreign pathogens or target cells. There are also other cell types that appear less commonly. They include but are not limited to circulating tumor cells (CTCs) in cancer, stem cells, and nucleated RBCs [35–38].

Collection of blood is performed by a phlebotomist and, depending on the type of downstream application, stored in tubes that may or may not contain additives. In scenarios that require the enrichment of blood cells, anticoagulating agents such as citrate phosphate dextrose adenine (CPDA) are typically added to prevent coagulation [39].

1.2.2.2 Bone Marrow

The bone marrow is a semi-liquid tissue that can be separated into two different forms: red and yellow marrow. The red marrow is involved in hematopoiesis, which is the production of blood cells such as RBCs and WBCs. Yellow marrow contains stromal cells and adipocytes, which produce growth factors that stimulate hematopoiesis. The type of marrow an individual possesses changes with age; infants possess close to 100% red marrow and the percentage decreases as they develop into adulthood, with an increase in yellow marrow. As hematopoiesis is a complex process with many different intermediate steps, the marrow is also a highly heterogeneous mix of different cells, ranging from hematopoietic stem cells to intermediate precursors and mature cells [17]. Therefore, in diseases such as leukemia, where one cell type in the marrow obtains uncontrolled growth,

it disrupts normal hematopoiesis and the patient is unable to replenish the other cell types [40, 41]. This can be easily diagnosed through a bone marrow examination, where the marrow is extracted, stained, and examined under the microscope. Because of its semi-liquid nature, bone marrow can be extracted either as an aspirate or a solid biopsy [42]. Harvesting bone marrow is also a method of isolating stem cells that can be used for treating leukemia through a bone marrow transplant [43].

1.2.2.3 Placental or Umbilical Cord Blood

Similar to the bone marrow, placental or umbilical cord blood is another rich source of stem cells that can be utilized for regenerative medicine [44, 45]. However, placental or umbilical cord blood can only be harvested from the placenta or umbilical cord immediately after birth. The collection of cord blood is done using either a syringe or blood collection bag. Unwanted RBCs are removed by methods such as density centrifugation. The stem cells are then enriched from the blood (described further in Section 1.3), mixed back with the donor's plasma, and cryopreserved in liquid nitrogen [46].

1.2.2.4 Urine

Urine is another important clinical sample that is routinely processed in the clinic. Mainly made up of water, urine is a by-product of the waste removal process by the kidneys and contains a variety of entities, both cellular and noncellular. Examples of the molecules that can be found in urine include protein, urea, chloride, or glucose, to name a few. Cells can also be observed in urine but their presence is limited, and detectable cells in the urine may indicate an underlying medical condition. Examples can include RBCs, epithelial cells (for bladder cancer detection), stem cells, and bacteria [47–50]. Urine is typically collected in a container and the collection process is relatively easy, which can be performed by the donors themselves. If the urine is to be used for cytology, the specimen is sent to the clinical laboratory as soon as possible or stored at 4 °C until then [49].

1.2.2.5 Cerebrospinal Fluid (CSF)

The main function of the CSF is to act as a physical and physiological buffer that acts to prevent physical trauma to the brain. The existence of the blood–brain barrier helps keep the CSF as a sterile environment and only allows substance(s) such as glucose or hormones from the blood to enter the brain while keeping pathogens out. However, this barrier can be compromised during diseases such as meningitis and can induce a change in the physical appearance of CSF as well as the appearance of immune cells [51]. In the cancer of the central nervous system, the CSF may also contain cancer cells. These can be identified through a lumbar puncture to harvest CSF followed by cytology to examine the CSF under the microscope

[52–54]. Similarly, the presence of tumor cells in the CSF may also indicate metastasis to the brain in other cancers such as breast cancer [55].

1.2.2.6

Saliva

Saliva is found secreted within the oral cavity and functions not only as a form of lubrication but also as an antibacterial agent. It contains many different proteins such as amylase, lysozymes, and immunoglobulins. The presence of lysozymes and immunoglobulins helps protect against bacterial infection, although they do not eliminate all bacterial flora within the oral cavity. Therefore, examining the types of bacterial cells found within saliva can be used to diagnose for pathogenic infections such as *Helicobacter pylori*, the etiological agent for gastric ulcers and cancer [56–58]. The collection of saliva is typically through expectoration [59].

In summary, there are many different types of clinical samples, each with their specific uses and properties. Furthermore, they consist of a heterogeneous mix of cellular and noncellular materials. In the following section, we describe how different types of tissues are processed after collection as well as some methods of cell enrichment.

1.3 Sample Processing and Conventional Methods of Cell Enrichment

Once a clinical sample has been harvested from a donor or animal after a surgical procedure, there is a need to process the sample for downstream applications. In this section, we aim to provide the reader with an introduction to the general procedure of sample processing and the conventional methods of cell enrichment.

1.3.1 Processing Solid Clinical Samples

If a piece of solid tissue harvested after resection is directed to a pathologist for examination, it is typically placed in a fixative such as 10% buffered formalin and sent to gross room, where the fixed tissue is further processed, assigned identification numbers, and sliced into sections to facilitate paraffin embedding. To embed the tissue, it is first immersed into liquid paraffin and the paraffin cooled to form a block of solid paraffin-containing tissue. Thin slices of the paraffin-embedded tissue are made using a microtome, and these slices are placed on a glass slide for histological staining. Finally, the stained samples are examined by the pathologist, and the findings are reported back to the clinician [60].

If fresh tissue from a clinical sample is required, it can be acquired from a clinical sample before being placed in fixative. This can be done in a variety of ways, such as cutting a piece from the specimen or performing an *ex vivo* biopsy on the specimen [61–63]. However, it is also important that the sampling process does not interfere with the downstream pathological assessment of the disease [60].

Before enrichment of cells can occur from solid tissue, the sample has to be first dissociated into a solution of cell suspension through a combination of physical and enzymatic approaches. The tissue is first cut into small pieces through the use of a sharp object such as a surgical blade. Next, enzymes such as trypsin or collagenase are added to the tissue to digest cellular interactions between cells as well as to break down the extracellular matrix [63–65]. Alternatively, an enzyme-free approach has been described in which the tissue of interest is dissociated through the use of spinning microblades. After physical and enzymatic dissociation, the minced tissue is passed through a filter or strainer that serves to further facilitate the separation of cell clusters to a single-cell suspension [66].

1.3.1.1 Processing Liquid Samples

Unlike solid clinical samples, liquid samples do not require any dissociation of the sample into single cells before enrichment. Instead of enzymes, other additives such as protease inhibitors or inhibitors of coagulation may be added to prevent loss of protein or prevent coagulation that may negatively affect the downstream process. The type of additives used depends on the type of clinical sample; for urine samples, additives such as boric acid, acetic acid, or protease inhibitors can be added [49, 67], whereas no additives are recommended for samples such as CSF [68, 69]. Many different additives are available for blood and each additive is chosen based on the portion of the blood that is required [70]. Furthermore, if there are different tests to be carried out on the harvested blood and each test requires a specific additive, there is a specific order to which tubes are used first [71].

1.3.2 Cell Enrichment

After processing, cell enrichment from clinical samples can be carried out using positive and/or negative enrichment methods. Positive enrichment involves actively capturing and isolating the cells of interest, while negative enrichment involves depleting the sample of any unwanted cells. These two strategies can be implemented together or on separately. In this section, we discuss some of the positive and negative enrichment techniques commonly used.

1.3.2.1 Laser Capture Microdissection (LCM)

Laser capture microdissection (LCM) was first introduced in 1996 but has rapidly gained acceptance as a powerful tool for cell enrichment [72, 73]. As described in Section 1.2.1.1, solid clinical samples are usually a mix of different types of cells, and LCM is widely used for isolating pure populations of cells from this heterogeneous mix. LCM was first used to harvest pure populations of cells from histological slides of tissue samples. The region of interest within the histology slide is first identified visually and marked on the instrument. Next, a transfer

film is pressed onto the slide and a laser beam is focused onto the region of interest, activating the transfer film that attaches to the cells in the process. Once the transfer film is lifted off the histology slide, any cells found in the region of interest are transferred along as well [72], resulting in a positive enrichment of cells. LCM can also be used to negatively enrich for cells by removing any unwanted cells [73]. Some disadvantages of LCM include the requirement for visual identification of the region of interest as well as the fact that most of the time, harvested cells from histology slides are no longer viable after the fixing and staining process, limiting any *in vivo* applications of harvested cells. However, LCM platforms have been adapted to isolate living cells from culture dishes and future advancements in technology may allow LCM to be used on fresh clinical samples [74].

1.3.2.2 Density Gradient Centrifugation

Density gradient centrifugation is a convenient method of enriching for cells based on their different densities and is most commonly used as a form of bulk enrichment for blood cells. As different blood cells have different buoyant densities, separation based on density can be easily achieved through the use of a density gradient created using substrates such as Ficoll or Percoll (Figure 1.2) [76–79]. First, the blood is diluted with a buffered solution such as phosphate-buffered saline (PBS) and added onto the density gradient solution, followed by centrifugation to separate the cells into different fractions. Depending on the solution used, the user may be able to obtain different fractions, which can be harvested through aspiration [80]. Separation based on density has also been applied as a pre-enrichment step in the isolation of cells from solid tissue (processed into single-cell suspension) and even stem cells from cord blood [81, 82]. Although density gradient centrifugation is an efficient way of separating cells, it often lacks specificity and is usually only used as a pre-enrichment step before more specific downstream enrichment protocols.

1.3.2.3 Fluorescence-Activated Cell Sorting (FACS)

There are a variety of highly specific methods of cell enrichment available and we describe some well-known examples in this section. Fluorescence-activated cell sorting (FACS) is currently one of the most effective methods of enriching for a specific subpopulation of cells from any sample (Figure 1.2). FACS operates based on biological and engineering principles. First, fluorescently labeled antibodies that recognize a specific protein unique to the cell of interest is added to a liquid suspension containing a heterogeneous mix of cells. As a result, only the cells of interest within this mix are labeled with the fluorophore. Next, the sample is loaded onto the FACS machine where the liquid sample is focused into a stream of single cells via hydrodynamic focusing, followed by ultrasound sonication to generate single droplets of single cells. These droplets are further exposed to a focused laser beam that is emitting at the excitation wavelength of the fluorophore conjugated to the antibody. If the fluorophore is present in the droplet, it gets

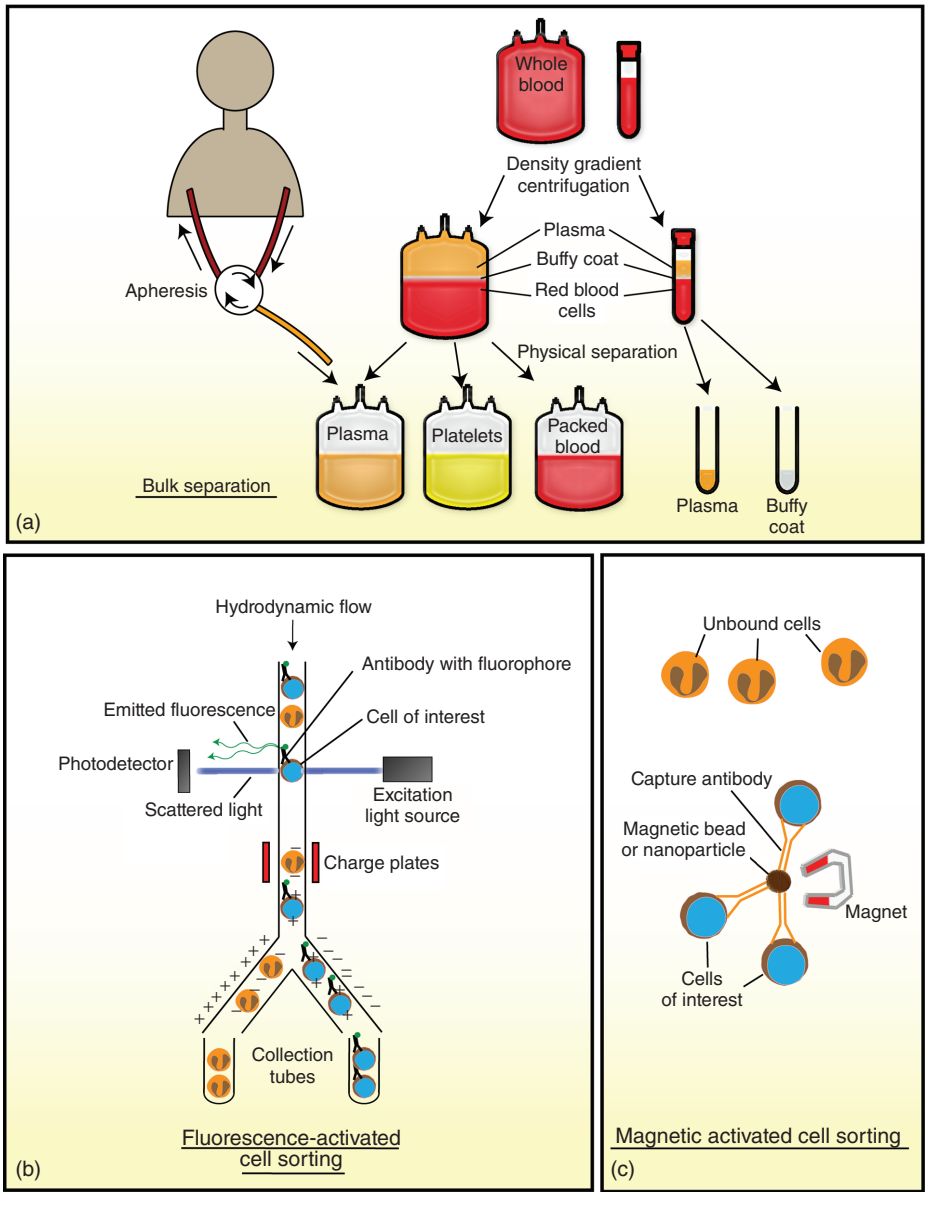

Figure 1.2 Schematic of conventional methods of blood processing. (Reproduced from Ref. [75] with permission of John Wiley and Sons.) Bulk separation of blood components by density gradient centrifugation (a); fluorescence-activated cell sorting (b); and magnetic activated cell sorting (c).

excited and starts to emit fluorescence that will be detected by a photodetector. Once a signal is recognized by the photodetector, an electrical pulse is generated that confers a charge to the droplet, allowing the flow path of the charged droplet to be easily manipulated with an electric field. The electric field deflects the path of the charged droplet away from the uncharged droplets, thus enabling efficient separation between the two [83]. FACS holds the advantages of being sensitive as well as specific, and offers positive cell enrichment. Furthermore, it is possible to analyze cell population at the same time as collection, allowing the user to determine the purity of the collected fraction. However, these instruments are often bulky, expensive, and time consuming to operate.

1.3.2.4 Magnetic Activated Cell Sorting (MACS)

Magnetic activated cell sorting (MACS) is an alternative to FACS as a method of cell enrichment. MACS and FACS are similar in that they both employ the use of biological and engineering principles to enrich for cells. However, unlike FACS, MACS makes use of a magnetic field as well as magnetic microbeads to aid in separation (Figure 1.2). First, the antibody recognizing a specific antigen on the surface of the cell of interest is conjugated to a magnetic microbead. This microbead-antibody is next added to the clinical sample and binds to any cells expressing the protein. This mix is passed through a column subjected to a strong magnetic field, which attracts and immobilizes any cell labeled with the microbead-antibody. Cells that are not labeled pass through the column and can be collected for further downstream applications. The cells immobilized on the column can be harvested by removing the magnetic field and subsequent wash steps [84]. MACS is a powerful tool that allows for positive and negative cell enrichment from a clinical sample at the same time. The process is also less harsh on the cells, allowing for viable cells to be harvested. However, real-time analysis of the purity of collected cells is not possible and will have to be confirmed by other methods such as flow cytometry. In addition, the isolation of rare cells may pose a challenge using this system [84].

1.3.2.5 CellSearch™

The CellSearch® system is the only US Food and Drug Administration (US FDA) approved device specialized for the enrichment of CTCs from cancer patients. As mentioned in Section 1.2.2.1, the presence of CTCs in the blood is associated with metastatic cancer, and the detection and enrichment of these cells possess great diagnostic and research value [37, 38, 85]. Before CTCs can be isolated from the blood, the sample has to be first processed as described in Section 1.3.2.2 through centrifugation to separate RBCs from the plasma and other cells. The plasma fraction containing cells are then ready for CTC isolation. The CellSearch system operates in a two-part manner. First, isolation of CTCs is performed using iron nanoparticles coated with antibodies toward EpCAM (a marker of epithelial cells) that are used to capture CTCs under a magnetic field. After capture,

fluorescent-labeled antibodies are used to stain for cell surface markers such as cytokeratin (CK) 8, 18, or 19 (marker of epithelial cells) and CD45 (marker of hematological cells) to distinguish between CTCs and non-CTCs. Images of the labeled cells are obtained and as a majority cancer cells are epithelial in origin, any cells positive for CK8, 18, or 19 are classified as CTCs and cells labeled positive for CD45 are classified as non-CTC [86]. The CellSearch system is a highly specialized device using specific surface markers to the isolation and detection of a CTC. However, due to its high specificity, it may miss out on cancer cells that do not express EpCAM, or CK8, 18, or 19. This is a real possibility as cancer cells are known to lose their epithelial phenotype during metastasis through a process known as *epithelial mesenchymal transition* (EMT) [85].

Although current tools of cell enrichment are capable of isolating desired cells from a heterogeneous mix, each has their disadvantages, such as lack of sensitivity or high cost of operation. In recent years, microfluidics has been rapidly gaining acceptance as a cheaper yet efficient approach to cell enrichment. In the remainder of this chapter, we discuss some of the fluidic principles as well as the microdevices used.

1.4 Microscale/Nanoscale Devices for Cellular Enrichment

The accessibility of microfabrication techniques in recent years has facilitated the advent of micro- or nanoscale devices capable of manipulating fluid flow at the microlevel (microfluidics). Such manipulation of fluids at the microlevel generates unique fluid behaviors that can be used for cell capture. Furthermore, the potential to incorporate additional physical as well as biological mechanisms together with these devices makes microfluidics a powerful alternative to conventional methods of enrichment. In this section, we describe several microfluidic devices used in cellular enrichment, their underlying principles, and how they can be combined with other biological or physical phenomena. Specifically, we describe (i) filtration, (ii) hydrodynamic mechanisms, (iii) surface treatments, (iv) magnetophoresis, (v) electrical methods, and (vi) acoustophoresis.

1.4.1 Filtration Approaches

Cellular enrichment using microfluidic filtration devices is an attractive route to pursue as they are relatively inexpensive, label-free, and capable of processing large sample volumes in a short time. Devices that incorporate physical filters in them typically involve a thin porous polydimethylsiloxane (PDMS) membrane with micrometer pore sizes [87]. Alternatively, structures such as micropillars can be incorporated into a microchannel acting as physical sieve to trap cells (Figure 1.3a) [88]. One major shortcoming to using a microfluidic filtration device alone lies with the fact that clogging of the filters often occurs, leading to

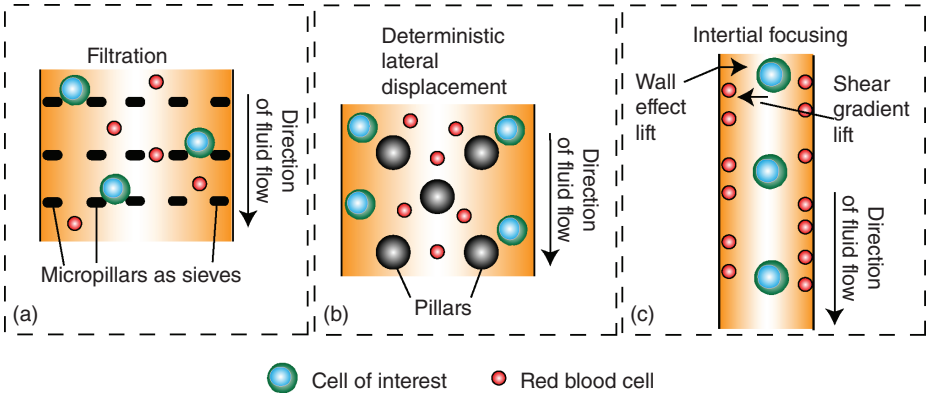

Figure 1.3 Schematic of different applications of cell isolation based on cell size. (a) Filtration methods typically include micropillars that act as sieves to trap cells. (b) Deterministic lateral displacement involves pillars placed in the channel that serves to deflect larger cells to the side of the channel while concentrating smaller cells in the center. (c) Inertial focusing is dependent on wall effect and shear gradient lifts that act to move larger cells to the center and smaller cells to the wall.

device failure. To circumvent this shortcoming, other features can be added to minimize clogging. One such example used featured a microfiltration membrane sandwiched between two layers of PDMS embedded with microchannels and microvalves. The incorporation of microchannels and microvalves enabled users to alternate between sample loading and washing/harvesting, thus preventing clogging of the device. Such a device has been successfully used to isolate larger Hematopoietic stem cells (HSCs) from other smaller cells in the bone marrow with relatively high efficiency, purity as well as viability [89]. Filtration devices can also be combined with biological methods such as the use of antibodies to enhance the specificity of enrichment. One application involved antibodies conjugated to microbeads, which effectively increased the size of the cell they are bound to. This increase in size trapped the cells of interest on the membrane while allowing other contaminating cells to flow out, resulting in a pure sample [87].

1.4.2 Hydrodynamic Mechanisms

As its name suggests, hydrodynamics depend on the manipulation of liquid flow and is a fairly popular method used in microfluidics for the enrichment of cells from a liquid suspension based on the physical and mechanical properties of the cell. There are many different possible applications of hydrodynamics: One example involved the use of sheath flow to first focus a sample of processed mouse heart tissue (as discussed in Section 1.3.1) consisting of a suspension of cardiomyocytes, fibroblasts, and other cells into a stream of single cells.

This stream was next directed to pass through an array of pillars which, upon collision with cells, would tend to deflect larger cells toward the side of the device (Figure 1.3b). This approach, also known as deterministic lateral displacement (DLD), resulted in the enrichment of larger cardiomyocytes from the sample with little loss of cell viability [90].

The purpose of placing an array of pillars in the path of a stream is not only to generate DLD but having an array of pillars also increases the effective surface area of interaction between the device and the liquid sample. This can be combined with biological mechanisms such as coating antibodies onto the pillars to increase the specificity and yield of capture. This setup was demonstrated to be effective in enriching for stem cells from the epidermis of animals [91]. In this study, enrichment was done using two devices with an array of pillars each, arranged in series. The first device was coated with an antibody specific for a marker found on non-stem cells and served as a negative enrichment device. The second device in the series was coated with an antibody specific for a stem cell marker and served as a positive enrichment device. Combining the two devices allowed for rapid isolation of stem cells from processed mouse epidermis with improved viability for downstream applications [91].

Inertial focusing is another mechanism of cell enrichment based on hydrodynamic principles and can be performed without the need for microstructures such as pillars. As a stream of liquid flows through a rectangular microchannel, different lift forces such as wall effect and shear-gradient lift force are generated. These forces serve to position objects within the microchannel based on size (Figure 1.3c) [92, 93]. Devices that employ inertial focusing have been used to separate cells from clinical samples based on size and other physical properties. In one study aimed at enriching CTCs from blood, the authors designed a device consisting of a single microchannel of varying width as well as expansion arrays along the device. As the microchannel width decreases along the device, it serves to focus larger CTCs to the center of the channel while forcing the smaller RBCs to the wall. The incorporation of the expansion arrays along the device acts as a form of equilibration for the cells' migration path. Using this device, viable cancer cells could be enriched from blood spiked with cancer cells [94]. Another device design used for enriching adrenal progenitor cells from the adrenal cortex involved an inlet followed by a rectangular microchannel of constant width with several outlets for cell collection. After tissue processing, the cell suspension was passed through the device that separated the heterogeneous cell suspension into progenitor (single cells) or differentiated cells (clumps of cells) within the microchannel [95]. Other possible applications of inertial focusing in cell enrichment include Dean flow fractionation, which involves the use of a spiral microchannel to generate additional Dean forces as fluid flows through the device in a spiral motion. This device acts as a centrifuge and is capable of separating different cells such as CTCs apart from normal hematological cells based on size [96, 97].

1.4.3 Surface Treatments

Functionalizing a surface for enhancing subsequent cell enrichment is an approach that can be incorporated with any microfluidic device. There are many surface treatment methods that are physical, biological, or a combination of both. In addition, PDMS used in constructing microfluidic devices can be easily manipulated through molding to introduce any new surface features. Examples of surface treatments in microfluidic devices include the addition of antibody-coated nanostructures such as pillars to the surface of the microchannel that acts to increase the effective area of interaction between cell and antibody. Other surface treatment methods such as adding herringbone structures serve to induce chaotic mixing as the sample flows over them, increasing the rate of interaction between cells and the nanopillars. Devices incorporating these functionalities have been successfully used in the enrichment of CTCs from patient blood at a high capture efficacy [98, 99].

Other substrates such as glass can be treated and used for cell enrichment as well. A simple form of treating glass involved roughening the surface using reactive ion etching (RIE). When blood spiked with cancer cells was added to the rough surface, the cancer cells demonstrated a preference to attach to the rough surface over untreated surfaces. This method presents a potential mechanism of capturing cancer cells from blood without the need for antibodies [100].

1.4.4 Magnetophoresis

The application of a magnetic field to aid in cellular enrichment is easy to achieve and does not affect cell viability drastically, contributing to its widespread use both in the clinic and laboratory. Well-known examples of magnetophoretic-based cell enrichment procedures include MACS and the CellSearch™ system (described in Sections 1.3.2.4 and 1.3.2.5). Magnetophoretic approaches have also been increasingly used in microfluidic devices due to improved fabrication techniques that allow the generation of microscale paramagnetic beads. Furthermore, the ability to conjugate antibodies to these microscale beads allows for highly specific enrichment.

Magnetophoretic microfluidic devices have been successful in enriching for cells such as circulating endothelial cells, a marker of diseases such as cardiovascular disease or SLE, from the blood of patients [101]. In this study, the device comprised multiple fluidic channels arranged in a radial fashion on a disk. Each channel consisted of an inlet reservoir that had a magnet positioned over it and a waste reservoir connected directly after the inlet reservoir. Blood from patients was first incubated with fluorescently labeled antibodies specific for surface endothelial markers. Next, magnetic beads conjugated with an antibody specific for the fluorophore was added to the sample, loaded onto the disk. Finally, the disk was spun, resulting in a centrifugal force applied to the liquid sample. Under

the magnetic field, the magnetic beads would immobilize any cells that had the fluorescent antibody bound within the inlet reservoir while the centrifugal force forced unlabeled cells to flow into the waste reservoir [101].

1.4.5 Electrophoresis

There are several advantages of using electrophoresis for cell enrichment. First, biological cells found in tissue possess unique dielectric properties, making it possible to separate them based on charge without the need to label the target with antibodies, as required by FACS and MACS [102, 103]. Second, integrating an electrical field to a microfluidic device is relatively easy and controlling the electrical field itself can be done in a robust manner. Third, the ability to generate direct current (DC) or alternating current (AC) allows the generation of various types of electrical fields that can be employed for cell enrichment. However, electrophoresis is dependent on the ionic concentration of the sample solution, which poses a limitation [104].

An example of electrophoresis in cell enrichment featured an integrated microfluidic device incorporating tissue processing, labeling, and electrophoretic microfluidic enrichment of tumor cells from head and neck squamous cell carcinoma (HNSCC) tumor samples on a single device. The device used in this study consisted of a reservoir for tissue processing, an integrated cell strainer as well as a microchannel filled with low melting point (LMP) agarose hydrogel. Tissue samples from HNSCC patients were first placed in the reservoir and perfused with enzymes and fluorescent antibodies specific for certain tumor cell surface markers at 4 °C overnight. At such low temperatures, the LMP agarose hydrogel remained as a solid, keeping the tumor sample within the reservoir and preventing any leakage of cells into the microchannel. After the overnight incubation, the solution in the reservoir was replaced with a buffered solution and incubated at 37 °C. This increase in temperature increased enzyme activity, accelerating the breakdown of cells from tissue and partially melted the LMP agarose, facilitating the entry of dissociated cells into the microchannel. An electrical field was applied across the microchannel and electrophoresis of tumor cells along the microchannel was possible due to the presence of the buffered solution. Interestingly, cell mobility within the microchannel was affected both by size and the presence of antibodies, thus allowing for selective tumor cell enrichment [105].

Another popular method of using electrical fields to isolate cells includes dielectrophoresis (DEP). This strategy requires the application of a nonuniform electrical field that confers kinetic energy onto a polarizable object such as a cell. The resulting electrokinetic energy helps direct motion of the cell toward or away from the electrode, termed positive or negative DEP, respectively [106, 107]. Such an approach is ideal for cell isolation based on their intrinsic electrical properties, which can also be altered based on the ionic concentration of the solution it is in and has been successfully utilized in the isolation of the bacteria *Escherichia coli* from whole blood and CSF (Figure 1.4a) [108]. In this study, the authors

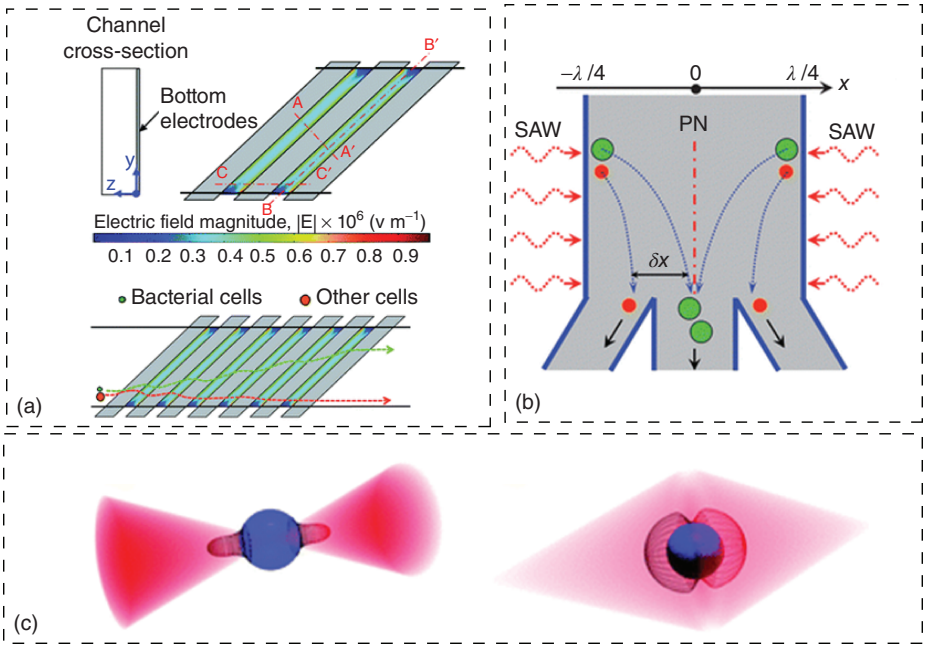

Figure 1.4 (a) Separation of bacterial cells from other cells using dielectrophoresis. The nonuniform electric field induces the bacterial cells to follow a different trajectory, thus enabling separation from other cells. (Reproduced from Ref. [108] with permission of The Royal Society of Chemistry.) (b) Separating particles of different sizes using surface acoustic waves. As particles such as cells flowing in the channel enters the path of the acoustic waves, the resultant force generated causes displacement of the particles. (Reproduced from Ref. [109] with permission of The Royal Society of Chemistry.) (c) Optical tweezers/traps: force fields are introduced by a single focused laser beam (left) or by two opposing laser beams on a dielectric object such as a cell. Color code: laser (red), dielectric object (blue), force field distribution (grid). (Reproduced from Ref. [110] with permission of The Royal Society of Chemistry.)

constructed a device with a cell separation module and a cell trapping module incorporating both negative (cell separation) and positive DEP (cell trapping). In addition, the authors included a buffer stream of lower ionic concentration flowing in parallel with the biological sample (blood or CSF). The low ionic concentration of the buffer stream altered the electrical properties of *E. coli*, which allowed the bacteria to separate away from the sample as it passes through the negative DEP module. The downstream positive DEP module served to trap the *E. coli*, allowing for further diagnostic analysis.

1.4.6
Acoustophoresis

The use of acoustophoresis in microfluidics typically employs the use of ultrasound, which generates a force capable of moving particles within the

microchannel. The mobility of the particle under acoustophoresis is dependent on the physical properties of the particle, making this technique usable when dealing with different sample media. Documented uses of acoustophoresis involved separating apoptotic cells from live ones. One such device involved a simple design consisting of two inlets – one for sample loading and the other for a buffer – a microchannel, two outlets, and a piezoelectric transducer to generate acoustic waves. The sample was first introduced as two sheath flows running parallel to the buffer stream. As cells transverse along the microchannel across the piezoelectric transducer, acoustic waves are generated that focus larger sized viable cells to the buffer stream while keeping smaller sized apoptotic cells in the sample stream. Viable cells in the buffer stream are directed to one of the two outlets for collection [111].

Other applications of acoustophoresis for cell isolation in microfluidics include standing surface acoustic wave or traveling surface acoustic wave [112]. To generate acoustic waves in these devices, interdigital transducers (IDTs) are first placed on the surface of a piezoelectric substrate. In the presence of an electric field, these IDTs in turn generate sound waves in the piezoelectric substrates that travel along the surface of the substrate. A typical surface acoustic wave microfluidic device possesses either one IDT on one side of a microchannel or two IDTs, each on either side. When a fluid flow bearing cells is introduced into the microchannel and enters the path of the sound wave, the resultant energy from the sound wave is capable of directing either the fluid flow or cell motion within the device (Figure 1.4b) [109, 113]. Surface acoustic waves have been successfully utilized to separate cancer cells from normal cells and have little negative impact on cell viability, making this approach compatible with further downstream cellular and molecular analysis [113].

1.4.7 Optical Tweezers/Traps

The use of a light source such as a laser to immobilize cells was first described in the late 1980s [114, 115]. The principle behind optical tweezers/traps involves the transfer of momentum from light photons to an object such as a cell as the cell passes through the narrowest part of focused laser beam [115]. Alternatively, two lasers can be arranged in opposing directions to create optical traps (Figure 1.4c) [110, 116]. The forces exerted are dependent on laser wavelength and intensity and are sufficiently strong to direct or stop the motion of the object. In cells, it has been demonstrated that the optical traps can trap cells based on the optical properties of the nuclei [117]. As a result, unlike FACS or MACS, which require prior antibody labeling, optical traps are ideal as a form of label-free cell isolation. This is crucial when attempting to isolate cancer cells that have an abnormal nucleus from a heterogeneous cell population mixed with normal cells and has been successfully used to isolate CTCs from blood [110, 118].

1.5 Conclusion

As discussed in this chapter, there are many forms of clinical samples, each possessing a unique complexity not only in the types of cells present, but in their clinical or research application as well. Currently, there exist several powerful tools capable of enriching cells in a highly sensitive and specific manner such as LCM, FACS, and MACS. However, each has its advantages and disadvantages, and there is much room for improvement. Microfluidics is fast becoming a viable alternative to such conventional methods due to their versatility and ease of use. Although still in their infancy stages, these devices have demonstrated great potential in matching conventional methods of cellular enrichment in areas such as cancer research. However, the majority of microfluidic devices designed for cancer research are optimized to handle liquid specimens and for the isolation of CTCs. As we discussed in this chapter, solid tissue specimens are also a rich source of clinical information, particularly for cancer. There is a need for devices that are designed to isolate cells from solid tissue in order to address tumor heterogeneity. With further improvements, it is possible that microfluidic devices will one day be accepted as a mainstream method of cellular enrichment.

References

1. Turk, J.L. (1993) Rudolf Virchow – father of cellular pathology. *J. R. Soc. Med.*, **86**, 688–689.
2. Hahn, S., Zhong, X.Y., Troeger, C., Burgemeister, R. *et al.* (2000) Current applications of single-cell PCR. *Cell. Mol. Life Sci.*, **57**, 96–105.
3. Stell, V.H., Norton, H.J., Smith, K.S., Salo, J.C. *et al.* (2007) Method of biopsy and incidence of positive margins in primary melanoma. *Ann. Surg. Oncol.*, **14**, 893–898.
4. Sebo, T.J., Bock, B.J., Cheville, J.C., Lohse, C. *et al.* (2000) The percent of cores positive for cancer in prostate needle biopsy specimens is strongly predictive of tumor stage and volume at radical prostatectomy. *J. Urol.*, **163**, 174–178.
5. Patel, A.R. and Jones, J.S. (2009) Optimal biopsy strategies for the diagnosis and staging of prostate cancer. *Curr. Opin. Urol.*, **19**, 232–237.
6. Scola, R.H., Pereira, E.R., Lorenzoni, P.J., and Werneck, L.C. (2007) Toxic myopathies: muscle biopsy features. *Arq. Neuropsiquiatr.*, **65**, 82–86.
7. Petri, M., Orbai, A.M., Alarcon, G.S., Gordon, C. *et al.* (2012) Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum.*, **64**, 2677–2686.
8. Lim, K.L., Abdul-Wahab, R., Lowe, J., and Powell, R.J. (1994) Muscle biopsy abnormalities in systemic lupus erythematosus: correlation with clinical and laboratory parameters. *Ann. Rheum. Dis.*, **53**, 178–182.
9. Theise, N.D. (2007) Liver biopsy assessment in chronic viral hepatitis: a personal, practical approach. *Mod. Pathol.*, **20** (Suppl. 1), S3–S14.
10. Manning, D.S. and Afdhal, N.H. (2008) Diagnosis and quantitation of fibrosis. *Gastroenterology*, **134**, 1670–1681.
11. Taniguchi, K., Okami, J., Kodama, K., Higashiyama, M. *et al.* (2008) Intratumor heterogeneity of epidermal growth factor receptor mutations in lung cancer and its correlation to the response to gefitinib. *Cancer Sci.*, **99**, 929–935.

12. Yancovitz, M., Litterman, A., Yoon, J., Ng, E. *et al.* (2012) Intra- and inter-tumor heterogeneity of BRAF(V600E))mutations in primary and metastatic melanoma. *PLoS One*, **7**, e29336.
13. Dalerba, P., Kalisky, T., Sahoo, D., Rajendran, P.S. *et al.* (2011) Single-cell dissection of transcriptional heterogeneity in human colon tumors. *Nat. Biotechnol.*, **29**, 1120–1127.
14. Heidenreich, A., Ohlmann, C.H., and Polyakov, S. (2007) Anatomical extent of pelvic lymphadenectomy in patients undergoing radical prostatectomy. *Eur. Urol.*, **52**, 29–37.
15. Dutton, J.J. (2013) Institutional review boards, declaration of Helsinki, and HIPAA regulations. *Ophthal. Plast. Reconstr. Surg.*, **29**, 335–340.
16. Frese, K.K. and Tuveson, D.A. (2007) Maximizing mouse cancer models. *Nat. Rev. Cancer*, **7**, 645–658.
17. Kerr, J.B. (1999) *Atlas of Functional Histology*, Mosby.
18. Hynes, R.O. and Naba, A. (2012) Overview of the matrisome – an inventory of extracellular matrix constituents and functions. *Cold Spring Harbor Perspect. Biol.*, **4**, a004903.
19. Bragulla, H.H. and Homberger, D.G. (2009) Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. *J. Anat.*, **214**, 516–559.
20. Trzpis, M., McLaughlin, P.M., de Leij, L.M., and Harmsen, M.C. (2007) Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. *Am. J. Pathol.*, **171**, 386–395.
21. Shapiro, L. and Weis, W.I. (2009) Structure and biochemistry of cadherins and catenins. *Cold Spring Harbor Perspect. Biol.*, **1**, a003053.
22. Kalluri, R. and Zeisberg, M. (2006) Fibroblasts in cancer. *Nat. Rev. Cancer*, **6**, 392–401.
23. Alonso, L. and Fuchs, E. (2003) Stem cells of the skin epithelium. *Proc. Natl. Acad. Sci. U.S.A.*, **100** (Suppl. 1), 11830–11835.
24. Ambler, C.A. and Maatta, A. (2009) Epidermal stem cells: location, potential and contribution to cancer. *J. Pathol.*, **217**, 206–216.
25. Umar, S. (2010) Intestinal stem cells. *Curr. Gastroenterol. Rep.*, **12**, 340–348.
26. Randolph, G.J., Angeli, V., and Swartz, M.A. (2005) Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat. Rev. Immunol.*, **5**, 617–628.
27. Park, J.E. and Barbul, A. (2004) Understanding the role of immune regulation in wound healing. *Am. J. Surg.*, **187**, 11S–16S.
28. Shortman, K. and Liu, Y.J. (2002) Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol.*, **2**, 151–161.
29. Howlader, N.N.A., Krapcho, M., Garshell, J., Neyman, N., Altekruse, S.F., Kosary, C.L., Yu, M., Ruhl, J., Tatalovich, Z., Cho, H., Mariotto, A., Lewis, D.R., Chen, H.S., Feuer, E.J., and Cronin, K.A. (2013) *SEER Cancer Statistics Review, 1975–2010*, National Cancer Institute, Bethesda, MD.
30. Compton, C., Fenoglio-Preiser, C.M., Pettigrew, N., and Fielding, L.P. (2000) American Joint Committee on Cancer Prognostic Factors Consensus Conference: Colorectal Working Group. *Cancer*, **88**, 1739–1757.
31. Epstein, J.I. (2010) An update of the Gleason grading system. *J. Urol.*, **183**, 433–440.
32. DeMarzo, A.M., Nelson, W.G., Isaacs, W.B., and Epstein, J.I. (2003) Pathological and molecular aspects of prostate cancer. *Lancet*, **361**, 955–964.
33. Bain, B.J. (2006) *Blood Cells a Practical Guide*, Blackwell, Malden, MA, Oxford.
34. Bledzka, K., Smyth, S.S., and Plow, E.F. (2013) Integrin alphaIIbbeta3: from discovery to efficacious therapeutic target. *Circ. Res.*, **112**, 1189–1200.
35. Greenbaum, A.M. and Link, D.C. (2011) Mechanisms of G-CSF-mediated hematopoietic stem and progenitor mobilization. *Leukemia*, **25**, 211–217.
36. Wachtel, S.S., Shulman, L.P., and Sammons, D. (2001) Fetal cells in maternal blood. *Clin. Genet.*, **59**, 74–79.
37. Liu, M.C., Shields, P.G., Warren, R.D., Cohen, P. *et al.* (2009) Circulating

tumor cells: a useful predictor of treatment efficacy in metastatic breast cancer. *J. Clin. Oncol.*, **27**, 5153–5159.
38. de Bono, J.S., Scher, H.I., Montgomery, R.B., Parker, C. *et al.* (2008) Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin. Cancer Res.*, **14**, 6302–6309.
39. Vaught, J.B. (2006) Blood collection, shipment, processing, and storage. *Cancer Epidemiol. Biomarkers Prev.*, **15**, 1582–1584.
40. Faderl, S., Talpaz, M., Estrov, Z., and Kantarjian, H.M. (1999) Chronic myelogenous leukemia: biology and therapy. *Ann. Intern. Med.*, **131**, 207–219.
41. Cortes, J.E. and Kantarjian, H.M. (1995) Acute lymphoblastic leukemia. A comprehensive review with emphasis on biology and therapy. *Cancer*, **76**, 2393–2417.
42. Lee, S.H., Erber, W.N., Porwit, A., Tomonaga, M. *et al.* (2008) ICSH guidelines for the standardization of bone marrow specimens and reports. *Int. J. Lab. Hematol.*, **30**, 349–364.
43. Rao, M., Ahrlund-Richter, L., and Kaufman, D.S. (2012) Concise review: cord blood banking, transplantation and induced pluripotent stem cell: success and opportunities. *Stem Cells*, **30**, 55–60.
44. Broxmeyer, H.E., Gluckman, E., Auerbach, A., Douglas, G.W. *et al.* (1990) Human umbilical cord blood: a clinically useful source of transplantable hematopoietic stem/progenitor cells. *Int. J. Cell Cloning*, **8** (Suppl. 1), 76–89; discussion -91.
45. Cairo, M.S. and Wagner, J.E. (1997) Placental and/or umbilical cord blood: an alternative source of hematopoietic stem cells for transplantation. *Blood*, **90**, 4665–4678.
46. Badowski, M.S. and Harris, D.T. (2012) Collection, processing, and banking of umbilical cord blood stem cells for transplantation and regenerative medicine. *Methods Mol. Biol.*, **879**, 279–290.
47. Zhou, T., Benda, C., Dunzinger, S., Huang, Y. *et al.* (2012) Generation of human induced pluripotent stem cells from urine samples. *Nat. Protoc.*, **7**, 2080–2089.
48. Clark, P.E., Agarwal, N., Biagioli, M.C., Eisenberger, M.A. *et al.* (2013) Bladder cancer. *J. Natl. Compr. Cancer Netw.*, **11**, 446–475.
49. Rabinovitch, A. and National Committee for Clinical Laboratory Standards (2001) *Urinalysis and Collection, Transportation, and Preservation of Urine Specimens: Approved Guideline*, National Committee for Clinical Laboratory Standards.
50. Bharadwaj, S., Liu, G., Shi, Y., Markert, C. *et al.* (2011) Characterization of urine-derived stem cells obtained from upper urinary tract for use in cell-based urological tissue engineering. *Tissue Eng. Part A*, **17**, 2123–2132.
51. de Vries, H.E., Kuiper, J., de Boer, A.G., Van Berkel, T.J. *et al.* (1997) The blood-brain barrier in neuroinflammatory diseases. *Pharmacol. Rev.*, **49**, 143–155.
52. Glantz, M.J., Cole, B.F., Glantz, L.K., Cobb, J. *et al.* (1998) Cerebrospinal fluid cytology in patients with cancer: minimizing false-negative results. *Cancer*, **82**, 733–739.
53. Bromberg, J.E., Breems, D.A., Kraan, J., Bikker, G. *et al.* (2007) CSF flow cytometry greatly improves diagnostic accuracy in CNS hematologic malignancies. *Neurology*, **68**, 1674–1679.
54. Seehusen, D.A., Reeves, M.M., and Fomin, D.A. (2003) Cerebrospinal fluid analysis. *Am. Fam. Physician*, **68**, 1103–1108.
55. Patel, A.S., Allen, J.E., Dicker, D.T., Peters, K.L. *et al.* (2011) Identification and enumeration of circulating tumor cells in the cerebrospinal fluid of breast cancer patients with central nervous system metastases. *Oncotarget*, **2**, 752–760.
56. Li, C., Musich, P.R., Ha, T., Ferguson, D.A. Jr., *et al.* (1995) High prevalence of *Helicobacter pylori* in saliva demonstrated by a novel PCR assay. *J. Clin. Pathol.*, **48**, 662–666.
57. Humphrey, S.P. and Williamson, R.T. (2001) A review of saliva: normal composition, flow, and function. *J. Prosthet. Dent.*, **85**, 162–169.

58. de Almeida Pdel, V., Gregio, A.M., Machado, M.A., de Lima, A.A. *et al.* (2008) Saliva composition and functions: a comprehensive review. *J. Contemp. Dent. Pract.*, **9**, 72–80.
59. Henson, B.S. and Wong, D.T. (2010) Collection, storage, and processing of saliva samples for downstream molecular applications. *Methods Mol. Biol.*, **666**, 21–30.
60. Samaratunga, H., Montironi, R., True, L., Epstein, J.I. *et al.* (2011) International Society of Urological Pathology (ISUP) Consensus Conference on Handling and Staging of Radical Prostatectomy Specimens. Working group 1: specimen handling. *Mod. Pathol.*, **24**, 6–15.
61. Walton, T.J., McCulloch, T.A., Rees, R.C., and Bishop, M.C. (2005) Obtaining fresh prostate cancer tissue for research: a novel biopsy needle and sampling technique for radical prostatectomy specimens. *Prostate*, **64**, 382–386.
62. Bell, W.C., Young, E.S., Billings, P.E., and Grizzle, W.E. (2008) The efficient operation of the surgical pathology gross room. *Biotech. Histochem.*, **83**, 71–82.
63. Lexander, H., Hellman, U., Palmberg, C., Auer, G. *et al.* (2006) Evaluation of two sample preparation methods for prostate proteome analysis. *Proteomics*, **6**, 3918–3925.
64. Phillips, H.J. (1972) Dissociation of single cells from lung or kidney tissue with elastase. *In Vitro*, **8**, 101–105.
65. Stingl, J., Emerman, J.T., and Eaves, C.J. (2005) Enzymatic dissociation and culture of normal human mammary tissue to detect progenitor activity. *Methods Mol. Biol.*, **290**, 249–263.
66. Boyd, Z.S., Raja, R., Johnson, S., Eberhard, D.A. *et al.* (2009) A tumor sorting protocol that enables enrichment of pancreatic adenocarcinoma cells and facilitation of genetic analyses. *J. Mol. Diagn.*, **11**, 290–297.
67. Zhou, H., Yuen, P.S., Pisitkun, T., Gonzales, P.A. *et al.* (2006) Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. *Kidney Int.*, **69**, 1471–1476.
68. Teunissen, C.E., Tum ani, H., Bennett, J.L., Berven, F.S. *et al.* (2011) Consensus guidelines for CSF and blood biobanking for CNS biomarker studies. *Mult. Scler. Int.*, **2011**, 246412.
69. Walts, A.E. and Strigle, S. (1995) Toward optimal use of the cytology laboratory: quality improvement and cerebrospinal fluid specimens. *Diagn. Cytopathol.*, **13**, 357–361.
70. Holland, N.T., Smith, M.T., Eskenazi, B., and Bastaki, M. (2003) Biological sample collection and processing for molecular epidemiological studies. *Mutat. Res.*, **543**, 217–234.
71. Calam, R.R. and Cooper, M.H. (1982) Recommended "order of draw" for collecting blood specimens into additive-containing tubes. *Clin. Chem.*, **28**, 1399.
72. Emmert-Buck, M.R., Bonner, R.F., Smith, P.D., Chuaqui, R.F. *et al.* (1996) Laser capture microdissection. *Science*, **274**, 998–1001.
73. Espina, V., Heiby, M., Pierobon, M., and Liotta, L.A. (2007) Laser capture microdissection technology. *Expert Rev. Mol. Diagn.*, **7**, 647–657.
74. Demou, Z.N. (2008) Time-lapse analysis and microdissection of living 3D melanoma cell cultures for genomics and proteomics. *Biotechnol. Bioeng.*, **101**, 307–316.
75. Yu, Z.T., Aw Yong, K.M., and Fu, J. (2014) Microfluidic blood cell sorting: now and beyond. *Small*, **10**, 1687–1703.
76. Pertoft, H. (2000) Fractionation of cells and subcellular particles with Percoll. *J. Biochem. Biophys. Methods*, **44**, 1–30.
77. Boyum, A. (1964) Separation of white blood cells. *Nature*, **204**, 793–794.
78. Grdina, D.J., Milas, L., Hewitt, R.R., and Withers, H.R. (1973) Buoyant density separation of human blood cells in renografin gradients. *Exp. Cell. Res.*, **81**, 250–254.
79. English, D. and Andersen, B.R. (1974) Single-step separation of red blood cells. Granulocytes and mononuclear

leukocytes on discontinuous density gradients of Ficoll-Hypaque. *J. Immunol. Methods*, **5**, 249–252.

80. Jaatinen, T. and Laine, J. (2007) Isolation of mononuclear cells from human cord blood by Ficoll-Paque density gradient. *Curr. Protoc. Stem Cell Biol.*, Chapter 2: Unit 2A 1, doi: 10.1002/9780470151808.sc02a01s1, 2A.1.1–2A.1.4.

81. Smedsrod, B., Pertoft, H., Eggertsen, G., and Sundstrom, C. (1985) Functional and morphological characterization of cultures of Kupffer cells and liver endothelial cells prepared by means of density separation in Percoll, and selective substrate adherence. *Cell Tissue Res.*, **241**, 639–649.

82. Charbord, P., Newton, I., Voillat, L., Schaal, J.P. *et al.* (1996) The purification of CD34 cells from human cord blood: comparison of separation techniques and cytokine requirements for optimal growth of clonogenic progenitors. *Br. J. Haematol.*, **94**, 449–454.

83. Bonner, W.A., Hulett, H.R., Sweet, R.G., and Herzenberg, L.A. (1972) Fluorescence activated cell sorting. *Rev. Sci. Instrum.*, **43**, 404–409.

84. Miltenyi, S., Muller, W., Weichel, W., and Radbruch, A. (1990) High gradient magnetic cell separation with MACS. *Cytometry*, **11**, 231–238.

85. Yu, M., Bardia, A., Wittner, B.S., Stott, S.L. *et al.* (2013) Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science*, **339**, 580–584.

86. Allard, W.J., Matera, J., Miller, M.C., Repollet, M. *et al.* (2004) Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin. Cancer Res.*, **10**, 6897–6904.

87. Chen, W., Huang, N.T., Oh, B., Lam, R.H. *et al.* (2013) Surface-micromachined microfiltration membranes for efficient isolation and functional immunophenotyping of subpopulations of immune cells. *Adv. Healthc. Mater.*, **2**, 965–975.

88. Mohamed, H., McCurdy, L.D., Szarowski, D.H., Duva, S. *et al.* (2004) Development of a rare cell fractionation device: application for cancer detection. *IEEE Trans. Nanobiosci.*, **3**, 251–256.

89. Schirhagl, R., Fuereder, I., Hall, E.W., Medeiros, B.C. *et al.* (2011) Microfluidic purification and analysis of hematopoietic stem cells from bone marrow. *Lab Chip*, **11**, 3130–3135.

90. Zhang, B., Green, J.V., Murthy, S.K., and Radisic, M. (2012) Label-free enrichment of functional cardiomyocytes using microfluidic deterministic lateral flow displacement. *PLoS One*, **7**, e37619.

91. Zhu, B., Smith, J., Yarmush, M.L., Nahmias, Y. *et al.* (2013) Microfluidic enrichment of mouse epidermal stem cells and validation of stem cell proliferation in vitro. *Tissue Eng. Part C Methods*, **19**, 765–773.

92. Di Carlo, D., Irimia, D., Tompkins, R.G., and Toner, M. (2007) Continuous inertial focusing, ordering, and separation of particles in microchannels. *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 18892–18897.

93. Zhou, J. and Papautsky, I. (2013) Fundamentals of inertial focusing in microchannels. *Lab Chip*, **13**, 1121–1132.

94. Bhagat, A.A., Hou, H.W., Li, L.D., Lim, C.T. *et al.* (2011) Pinched flow coupled shear-modulated inertial microfluidics for high-throughput rare blood cell separation. *Lab Chip*, **11**, 1870–1878.

95. Hur, S.C., Brinckerhoff, T.Z., Walthers, C.M., Dunn, J.C. *et al.* (2012) Label-free enrichment of adrenal cortical progenitor cells using inertial microfluidics. *PLoS One*, **7**, e46550.

96. Hou, H.W., Warkiani, M.E., Khoo, B.L., Li, Z.R. *et al.* (2013) Isolation and retrieval of circulating tumor cells using centrifugal forces. *Sci. Rep.*, **3**, 1259.

97. Warkiani, M.E., Khoo, B.L., Tan, D.S., Bhagat, A.A. *et al.* (2014) An ultra-high-throughput spiral microfluidic biochip for the enrichment of circulating tumor cells. *Analyst*, **139**, 3245–3255.

98. Wang, S., Liu, K., Liu, J., Yu, Z.T. *et al.* (2011) Highly efficient capture of circulating tumor cells by using nanostructured silicon substrates with

integrated chaotic micromixers. *Angew. Chem. Int. Ed.*, **50**, 3084–3088.

99. Stott, S.L., Hsu, C.H., Tsukrov, D.I., Yu, M. *et al.* (2010) Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 18392–18397.

100. Chen, W., Weng, S., Zhang, F., Allen, S. *et al.* (2013) Nanoroughened surfaces for efficient capture of circulating tumor cells without using capture antibodies. *ACS Nano*, **7**, 566–575.

101. Chen, K.C., Lee, T.P., Pan, Y.C., Chiang, C.L. *et al.* (2011) Detection of circulating endothelial cells via a microfluidic disk. *Clin. Chem.*, **57**, 586–592.

102. Markx, G.H. and Davey, C.L. (1999) The dielectric properties of biological cells at radiofrequencies: applications in biotechnology. *Enzyme Microb. Technol.*, **25**, 161–171.

103. Gabriel, C., Gabriel, S., and Corthout, E. (1996) The dielectric properties of biological tissues: I. Literature survey. *Phys. Med. Biol.*, **41**, 2231–2249.

104. Gascoyne, P.R. and Vykoukal, J. (2002) Particle separation by dielectrophoresis. *Electrophoresis*, **23**, 1973–1983.

105. Woods, J., Docker, P.T., Dyer, C.E., Haswell, S.J. *et al.* (2011) On-chip integrated labelling, transport and detection of tumour cells. *Electrophoresis*, **32**, 3188–3195.

106. Wang, M.W. (2012) A microfluidic device for separating erythrocytes polluted by lead (II) from a continuous bloodstream flow. *Electrophoresis*, **33**, 780–787.

107. Jones, T.B. (1995) *Electromechanics of Particles Cambridge*, Cambridge University Press, New York.

108. Park, S., Zhang, Y., Wang, T.H., and Yang, S. (2011) Continuous dielectrophoretic bacterial separation and concentration from physiological media of high conductivity. *Lab Chip*, **11**, 2893–2900.

109. Shi, J., Huang, H., Stratton, Z., Huang, Y. *et al.* (2009) Continuous particle separation in a microfluidic channel via standing surface acoustic waves (SSAW). *Lab Chip*, **9**, 3354–3359.

110. Dochow, S., Krafft, C., Neugebauer, U., Bocklitz, T. *et al.* (2011) Tumour cell identification by means of Raman spectroscopy in combination with optical traps and microfluidic environments. *Lab Chip*, **11**, 1484–1490.

111. Yang, A.H. and Soh, H.T. (2012) Acoustophoretic sorting of viable mammalian cells in a microfluidic device. *Anal. Chem.*, **84**, 10756–10762.

112. Ding, X., Li, P., Lin, S.C., Stratton, Z.S. *et al.* (2013) Surface acoustic wave microfluidics. *Lab Chip*, **13**, 3626–3649.

113. Franke, T., Braunmuller, S., Schmid, L., Wixforth, A. *et al.* (2010) Surface acoustic wave actuated cell sorting (SAWACS). *Lab Chip*, **10**, 789–794.

114. Ashkin, A. and Dziedzic, J.M. (1987) Optical trapping and manipulation of viruses and bacteria. *Science*, **235**, 1517–1520.

115. Ashkin, A., Dziedzic, J.M., Bjorkholm, J.E., and Chu, S. (1986) Observation of a single-beam gradient force optical trap for dielectric particles. *Opt. Lett.*, **11**, 288.

116. Guck, J., Ananthakrishnan, R., Mahmood, H., Moon, T.J. *et al.* (2001) The optical stretcher: a novel laser tool to micromanipulate cells. *Biophys. J.*, **81**, 767–784.

117. Perney, N.M., Horak, P., Hanley, N.A., and Melvin, T. (2012) The self-orientation of mammalian cells in optical tweezers – the importance of the nucleus. *Phys. Biol.*, **9**, 024001.

118. Landenberger, B., Hofemann, H., Wadle, S., and Rohrbach, A. (2012) Microfluidic sorting of arbitrary cells with dynamic optical tweezers. *Lab Chip*, **12**, 3177–3183.