

# Chapter 5

## Microfluidics-based Single Cell Analytical Platforms for Characterization of Cancer

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**Abstract** Individual cancer cells in a tumor are very diverse both genetically and functionally. Moreover, in many cancers, whether a tumor flourishes or dies after a given treatment depends on a small fraction of cells in the tumor, for instance, the cancer stem cells, rather than the bulk population. Traditionally, scientists only obtain averaged information from the entire population of cells in a bulk tumor, but it is critical to develop an effective and user-friendly platform capable of interrogating single cells in order to understand and treat the disease better. This chapter reviews recent progress of microfluidics-based tools for single cell analysis that are relevant for cancer characterization, as well as how nanotechnology may advance the analysis with improved signal responses. We hope that this general introduction may catalyze the adoption of these advanced single-cell analysis approaches for cancer studies.

**Keywords** Nanosensors • Circulating tumor cells • Genomic analysis • Enzymatic activities

### 5.1 The Importance of Single Cell Analyses for Cancer Characterization

Cells are defined as the basic functional unit of living organisms. They sense environmental stimuli, respond and adapt to the environmental changes chemically or physically in order to form and maintain the distinct tissues in complex biological organisms. Therefore, even cells of identical genetic identities can display a wide disparity of physical responses (such as cell morphologies) or chemical responses (such as RNA or protein expression level regulation). These asynchronous responses

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to the microenvironments, often due to random fluctuations, or noise in gene expression [1–3], make precise characterization of cells in a population difficult, especially when techniques with single cell sensitivity are not available. Hence, currently our understanding of cells are in many aspects limited by the inherent ensemble average obtained by analyzing bulk populations of cells, which does not necessarily precisely represent the individual behavior of each cell within a population.

While many population-level studies using bulk assays are presented, it remains ambiguous whether the population data may faithfully reflect the dynamic response of individual cells. In fact, cell-to-cell heterogeneity has been evidenced in many examples. For instance, the transcription events in mammalian cells are subject to random fluctuations and lead to large variation in messenger RNA (mRNA) copy numbers [4, 5]. Moreover, cell heterogeneity is observed to impact cell-fate decisions in mouse multipotent progenitor cells [6]. In particular, cancers are typically characterized by a very high level of cell-to-cell variation in regard to both morphological, physiological and genetic features [1, 7, 8]. The obvious reason for such heterogeneity is that cancers arise as a consequence of multiple and successive mutations in the genome of the cell leading to the generation of many different subpopulations of cells constituting the tumor. According to the cancer stem cell theory, which was presented a few decades ago, cancer initiates by the accumulation of mutations in stem cells [9]. This leads to the transformation of the stem cells into cancer stem cells, with self-renewal potential and the capability to differentiate into other types of cancer cells. Due to these characteristics, isolation and characterization of individual cells within a population of cancer cells (e.g. a tumor) are envisioned of particular value for understanding the origin and progression of this disease.

Especially as a predictive tool for individualized cancer treatment, single cell characterization may prove of tremendous value. Recent studies suggest that the drugs developed by measuring therapeutic responses from traditional bulk analysis, tend to suppress the growth of bulk tumor cells but not as effective in eliminating cancer stem cells (CSCs), where the relapse of tumor may occur from [10]. The different drug responses between bulk tumor and CSCs further demonstrate that the fidelity of bulk analysis is insufficient for the evaluation of antitumor treatments. Furthermore, a potential innate consequence of the heterogenic nature of tumor cells is great fluctuations in the amount/activity of cellular drug targets or important determinants of drug efficiency, such as growth rate or metabolic activity.

For example, the heterogeneity of prostate cancer poses a significant challenge to effective targeted therapy [11, 12], such as those involved introduction of a homologous double-stranded RNA (dsRNA), or RNA interference (RNAi) [13], simply because the individual cancerous cells respond very differently to the same treatment. Survival of only a few cancer cells after initial systemic treatment may result in recurrent tumors, which may be even more aggressive or chemoresistant than the initial tumor. Therefore, the identification of these rare cells may be imperial for tailoring treatment to the individual patient in the best possible way.

Finally, when it comes to detection of cancers for diagnostic purposes, the capture and registration of single tumor cells from the bloodstream may prove a powerful tool. Indeed, in combination with protocols for fast and reliable single-cell characteristics of captured cells, such techniques may pave the road for future diagnosis integrated with individually tailored treatment.

Previous development of microfluidics has shown great success in biomedical applications, but mainly centered on the analysis of targeting biomolecules from minute amount of samples, typically nanoliters to hundreds of picoliters. More recently, the exploitation of microfluidics for cell manipulation and analysis, taking advantages of the matching length scale of microfluidics and the size of individual cells (tens of micrometers) has increased. Of particular importance is the detection and analysis of diseased cells, which are typically rare among the whole cell population, difficult to detect and retrieve, and yet critical for accurate analysis of the biological processes. In this chapter, we intend to review the existing development of microfluidics-based tools for single cell analysis that are relevant for cancer characterization, as well as how nanotechnology may advance the analysis with improved signal responses. We hope that this chapter will give the audience a general introduction and catalyze the adoption of these advanced single-cell analysis approaches [2, 4, 14] for cancer studies.

## 5.2 Enrichment of Circulating Tumor Cells

### 5.2.1 *What Are Circulating Tumor Cells?*

Metastasis, the spread of cancer cells from the primary site to other organs in the body, represents the major cause of cancer-related patient death. Previous evidence suggests that the tumor cells are shed from the primary tumor at an early stage of metastasis development, break through the vascular wall, travel via the peripheral blood to sites distant from the primary tumor, and form a secondary tumor [15]. Cells escaping from the primary tumor, are called circulating tumor cells (CTCs). The significant role of CTCs in the metastatic spread of tumor has rendered them valuable biomarkers for both detection of the onset of cancer metastasis and clinical evaluation of treatment outcome. Moreover, detecting CTCs as a cancer marker is advantageous in the clinics because it makes noninvasive detection possible through capturing CTCs in a liquid biopsy, such as a blood sample. Recent evidence on how the CTCs may reflect the molecular features of the primary tumor cells further displays the importance of CTCs in cancer biology [15, 16]. For instance, the presence of mesenchymal markers on CTCs envisages more accurate prognosis than the expression of cytokeratins alone, implying that the currently used assays based on epithelial antigens may overlook the most aggressive subpopulation. However, CTCs are extremely rare and appear in very low concentrations down to one per millions of normal blood cells. Therefore, their detection remains a great challenge in cancer characterization [15, 17].

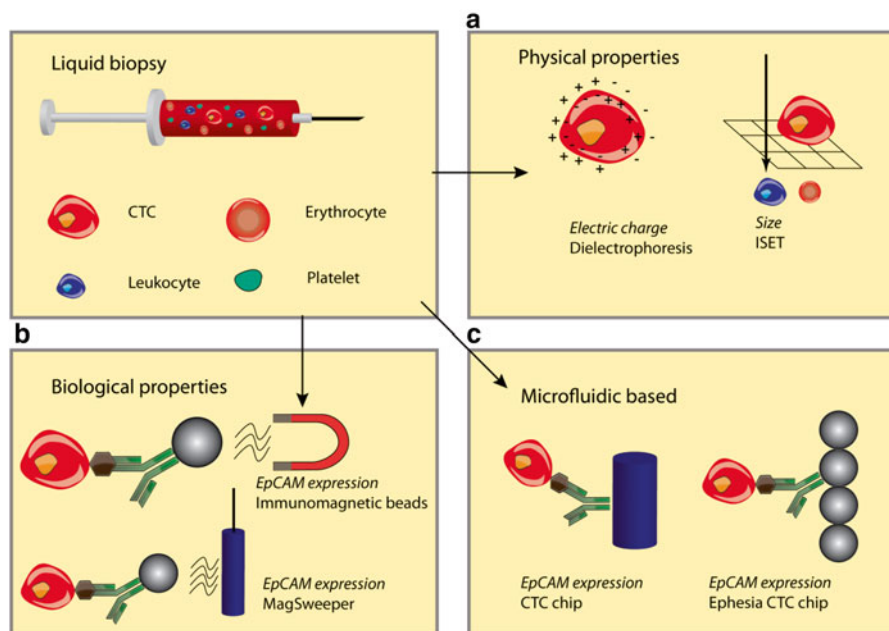
### 5.2.2 *State-of-Art*

CTC isolation is typically evaluated by many factors, including the capture efficiency (i.e. 100 % capture efficiency being isolation of all of the CTCs in the liquid biopsy, therefore, allowing identification of the cancer occurrence), isolation purity (i.e. 100 % isolation purity being isolation of only the CTCs, with no other cell types), isolation speed and the required sample volume. A large panel of macro-scale sorting techniques have been previously reported for CTC enrichment, such as immunomagnetic beads separation [18], laser scanning cytometry [19], fiber-optic array scanning technology (FAST) [20], as detailed in previously published reviews [18, 21]. In general, these approaches utilize the differences between CTCs and normal hematologic blood cells in physical (size, density, electric charges, deformability) or biochemical (surface protein expression, invasion capacity) properties, as illustrated in Fig. 5.1. For example, separation without labeling through the physical properties of the cells is adopted in the isolation by size of epithelial tumor cells (ISET) [22–24]. Dielectrophoretic field-flow fractionation (DEP-FFF) utilizes membrane resistance in combination with size to sort different responses to dielectrophoresis. Biochemical separation relies on immunological procedures using antibodies against tumor-associated antigens and common leukocytes antigens. For instance, the CellSearch<sup>®</sup> and the Ariol<sup>®</sup> select CTCs by utilizing magnetic beads coated with antibodies against genes that are highly expressed in CTCs [18, 21, 25]. Subsequently, the antigen-antibody complex is separated from the liquid phase *via* exposure to a magnetic field. However, many of the currently available approaches remain relatively ineffective in isolation efficiency with CTC identification in 50–90 % of patients, while 5–10 mL of sample volume is typically required [21]. Therefore, the search for sensitive, specific, and economical analytical techniques continues.

### 5.2.3 *Microfluidics Based CTC Enrichment*

Microfluidics based CTC enrichment has garnered considerable attention, due to the matching length scale of microfluidic channels to the cell sizes. Secondly, the micron-sized geometric features used in microfluidics greatly reduce the sample consumption. To date, microfluidics-based CTC enrichment has shown great promise by identification of CTCs in close to 99 % of patients [26], while requiring very minute sample size of 10  $\mu$ l [27].

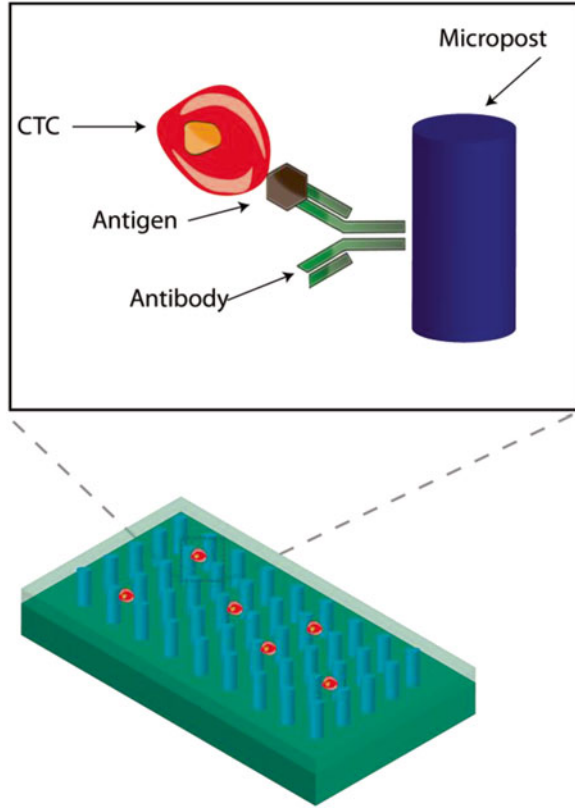
The very early demonstration of microfluidics based CTC enrichment is the so-called “CTC-chip” developed by Toner’s group [26]. The CTC-chip consists of an array of microposts coated with anti-EpCAM antibodies, as shown in Fig. 5.2, where the positive selection is implemented by the antibodies against the epithelial cellular adhesion molecule (EpCAM), relevant to epithelial growth and differentiation. Over expression of EpCAM has been observed in many human carcinomas including prostate, colon and rectum, breast, lung, esophagus, and pancreas.



**Fig. 5.1** Strategies to isolate circulating tumor cells (CTCs): Isolation of CTCs from normal hematologic blood cells, such as leukocyte, lymphocyte and erythrocyte, typically relies on dissimilar physical or biochemical properties of CTCs. (a) Separation by physical properties normally does not involve additional labeling, such as isolation by size of epithelial tumor cells (ISET), or the dielectrophoretic field-flow fractionation (DEP-FFF) that isolates cells by their responses to dielectrophoresis, which is determined by the size and membrane resistance. (b) Separation by biological properties: This category of separation usually involves immunological procedures which involve antibodies against tumor-associated antigens and common leukocytes antigens. The CTCs-specific antibodies are typically bound to micron-sized magnetic beads, which allow a separation when applying a magnetic field. (c) Microfluidics-based separation: Typical form of microfluidics-based CTC enrichment consists of microposts functionalized with antibodies specific to the surface-markers of tumor cells, such as epithelial cellular adhesion molecule (EpCAM). The microposts may be constructed by a solid structure, or by a pile of magnetic beads as recently demonstrated in the Ephesia CTC chip

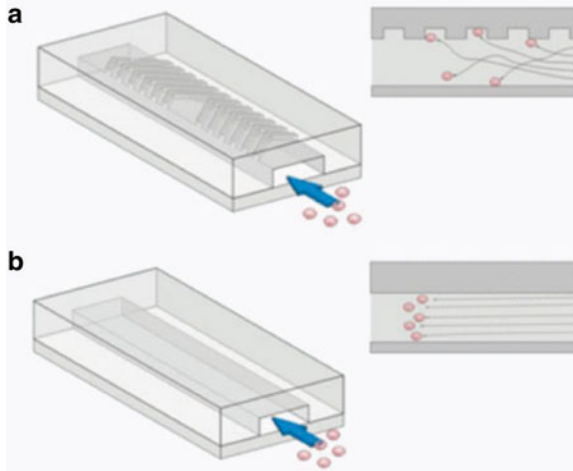
In contrast, hematologic cells do not express EpCAM [28, 29]. Hence, EpCAM appears as an effective cancer biomarker and an appropriate target molecules for CTCs enrichment in a liquid biopsy. Combining immunolabeling with controlled laminar flow conditions in the microfluidic chip, the CTC-chip has shown to successfully identify CTCs in peripheral blood from 99 % of patients carrying metastatic lung, prostate, pancreatic, breast, and colon cancer, or more precisely, 115 identifications out of 116 investigated samples [26]. The capture efficiency, as defined previously, is improved by two essential parameters operated in microfluidics: (a) Low flow speed: permitting cells to interact with the microposts for extended duration and, thus, increasing the likelihood of cells sticking to the posts and (b) Low shear stress: enabling the cells to flow through the channel with minimum physical

**Fig. 5.2** Typical design of the CTC-Chip: The microfluidic-based separation takes advantages of the comparable size of microfluidic channel and the cell sizes, that allows effective capture of CTCs. This chip usually consists of an array of micron-sized pillar posts functionalized by antibodies specific to CTCs. The microposts are used to disturb the flow-streamline and enhance cell-microposts interactions. Current development has centered on the design and production of microposts to scale up for large-scale clinical applications



distress. The CTC chip appears very gentle to the cells with a shear force of less than  $0.4 \text{ dyn/cm}^2$ . The low shear supplied in the CTC-chip even allows capture of T-24 cells, which have a relatively low expression of EpCAMs. Other than EpCAM, current characterization of CTCs is typically through immunostaining of the cells with markers such as KLK3 (prostate specific antigen) for prostate cancer or TTF-1 (thyroid transcription factor-1) for lung adenocarcinoma. Furthermore, to accelerate the isolation speed, Stoot et al. later demonstrated an automated system for prostate cancers with enrichment and quantitative analysis of positive CTCs for prostate specific antigen (PSA) [30].

As an alternative to the microposts based CTC-chip, an innovative geometric improvement termed the Herringbone-chip (HB-chip) [31], has been demonstrated to prime the enrichment of CTC. The chevrons, or the herringbones, as depicted in Fig. 5.3, function by disrupting the laminar flow, which would then enhance the mixing between streamlines and encourage the collisions of cells and the antibody-coated herringbone structures. The HB-chip has shown a 26.3 % improvement in capture efficiency compared to the previously discussed CTC-chip, along with significantly higher purity of the captured CTCs. Moreover, the use of transparent and chemically stable materials allows imaging of the captured cells with standard staining or assays such as Fluorescent In Situ Hybridization (FISH).



**Fig. 5.3** The Herringbone (HB)-Chip: Conventional design of CTC-Chip relies on laminar flow, which limits the interactions of target cells with surfaces, and the complex micropost structure is also challenging to scale up for high-throughput production. The HB-chip represents an alternative strategy, which takes advantages of the design of herringbones, hence the name. (a) The surface ridges help to break up streamlines, maximizing collisions between target cells and the antibody-coated walls. (b) As a comparison, there is a lack of mixing under low Reynolds number regime in traditional flat-walled design. Figure adapted from Ref. 31 with permission

Saliba et al. have recently developed a system, named Ephesia, in which they combine superparamagnetic beads with microfluidic technology [27]. In this platform, superparamagnetic beads are pre-functionalized with antibodies. When introduced into the chip, upon application of an external magnetic field, the beads would stack up due to dipole-dipole interactions, forming microposts out of stacks of beads. To reduce production cost and technical complexity, the magnetic pattern is generated through microcontact printing with water-based ferrofluid, or “magnetic ink”. Major benefits of this method, compared to the conventional design of CTC-chip, are the greatly reduced production cost and possible batch-functionalization of the magnetic beads. It is also of particular note that the proposed self-assembly process offers an aspect ratio beyond the most sophisticated nanofabrication techniques. In summary, the microfluidic technologies have emerged as an attractive micro-scale CTC isolation system. The unique features of fluidic mechanics at the micro- and nano-scale, such as channel dimensions, flow operated at the laminar flow regime, and surface area to volume ratio, have enabled improved capture efficiency and isolation purity of CTC. However, perhaps similar to the challenges for other microfluidics-based applications, it is necessary to validate the reproducibility and robustness of technology with extensive clinical relevant testing. Further, it is expected that the reduced technical complexity, together with low cost of production and testing, would encourage the widespread adoption of microfluidics-based CTC enrichment in the clinics. Audiences interested in the latest development of microfluidics-based CTC detection are referred to recently published reviews [32–34].

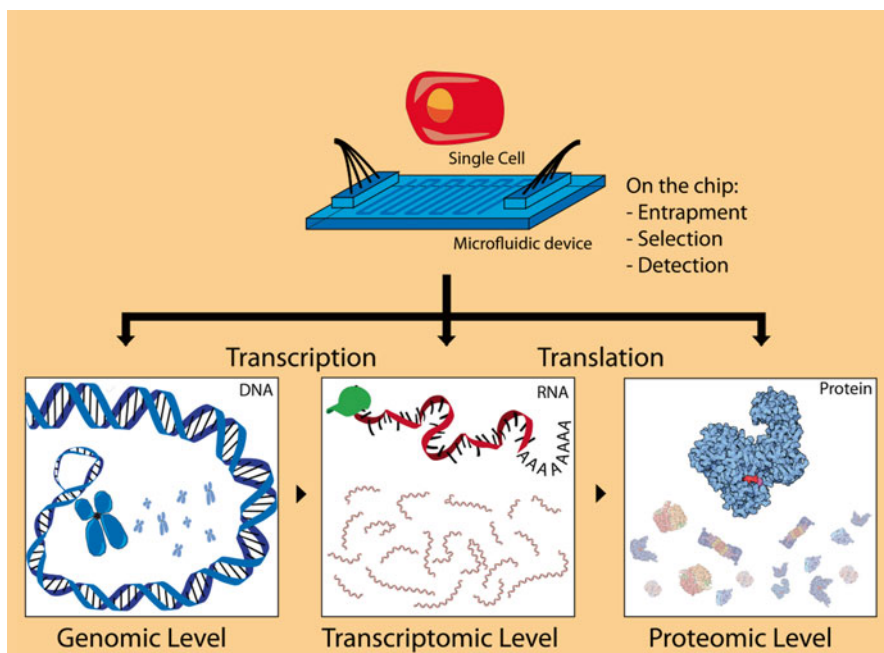


### 5.3 Nanoscaled Molecular Techniques for Analysis of Single Cells

Nanotechnology based molecular techniques, including nanoscale molecular manufacturing, nanosensors, and single molecule detection, represent a significant evolution towards investigation of cell population heterogeneity. As described above, single cell analysis and cell population heterogeneity investigation is necessary in order to elucidate how the rare cells may contribute to tumor development and treatment outcome. In the following section, we overview and comment on how nanoscaled molecular techniques may join the effort of microfluidics in single-cell analysis for the characterization of cancers from three perspectives: The genomic-, transcriptomic- and proteomic-level (as illustrated in Fig. 5.4).

#### 5.3.1 Single Cell Genome Analysis for Cancer Characterization

Cancers are typically caused by errors, or mutations, induced in the genome, which subsequently prompt the cells to malfunction, or grow uncontrollably. Such mutations can either be subtle genomic alterations (such as single base mutations) or gross



**Fig. 5.4** Overview of how microfluidic systems may assist in single cell analysis: High-throughput cell-based screens can benefit considerably from the unique liquid-handling capabilities offered by microfluidic systems



genomic alterations (such as deletions, translocations, insertions, rearrangement or even loss or gain of entire chromosomes) [35]. As the disease progresses, the evolutionary accumulation of cancer causing mutations result in a high degree of genetic diversity between or among cancer cells [36–38]. Furthermore, it is becoming established that CSCs, which account for only a minor part of the bulk tumor, exist in a variety of cancers, such as colon, ovarian, and small cell lung cancers [39]. CSCs are known to have a distinct gene expression phenotype, rendering them highly resistant to chemotherapeutic treatments [39–42].

Identification of genomic alterations at the single cell level are routinely accomplished today by standard cancer diagnostic techniques such as chromosome staining and FISH [43–45], or FISH based on nanoparticles-labeled probes [46]. These refined techniques are based on microscopic readouts, and can be used to identify large genomic alterations in single cells. In some cases FISH can even be used to detect Single Nucleotide Polymorphisms (SNPs) if the site and polymorphism is known [47]. In recent years, genomic sequencing has been adapted for single human cell genome analysis [48, 49]. This late arrival of DNA-sequencing as a tool for single cell analysis reflects the preceding barriers: (1) sufficiently sensitive sequencing techniques for single cell analysis have emerged not until the last decade, (2) the data obtained by sequencing is often very comprehensive and the analysis of which requires well-established bioinformatics tools and/or statistics as well as appropriate references. With this said, a combination with microfluidics might provide a unique means for isolation and/or enrichment of specific populations of cells, for example the CSCs.

As a pioneer in the full genome sequencing of single human cells using single cell based approaches, Wigler's group combined flow-sorting, Whole Genome Amplification (WGA) and next-generation sequencing for the investigation of single breast cancer cells [49]. More than 400 cells were sequenced with around 6 % genome coverage of each cell. Due to the suboptimal genome coverage (~6 %), sequencing data was clustered in 54 kilo base (kb) sized "bins", to obtain proper statistical significance. These bins were then used to determine the copy numbers of genomic areas mapped to the healthy genome, which enabled the analysis of the evolutionary history of the cancer. Another example of genome sequencing of single cells is provided by Frumkin and colleagues [48] however, this was done without the use of any microfluidics or flow based techniques. In this study, cancer lineage relations were investigated by cutting out single tumor cells from tissue sections of mouse lymphoma by microdissection. Around 50 single cells were genotyped using Sanger sequencing and data was used to produce a "lineage tree" for the analyzed cells populating the tumor.

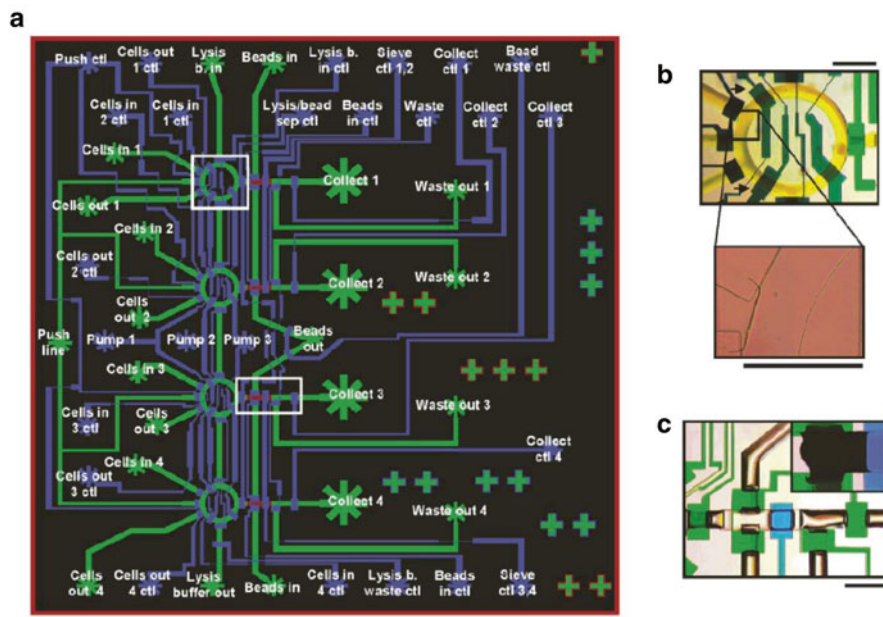
These studies provide a demonstration of the need of single cell sequencing in characterization of cancer, along with pros and cons of the utilized approaches. For example, microdissection on tissue samples allows the selection of specific cells. However, microdissection holds a general limitation in relation to number of analyzed cells: ~50 in Frumkin's study compared to >400 in the study by Navin et al. using a flow based single cell analysis. Moving forward, microfluidics based approaches are expected to ease the handling of sample and enhance the enrichment efficiency prior to the genome analysis. Moreover, the automation offered by

microfluidics also makes the experiments less prone towards human errors, such as the biased selection of cells by manually picking, and contamination of sample by human handling.

### ***5.3.2 Single Cell Transcription Analysis for Cancer Characterization***

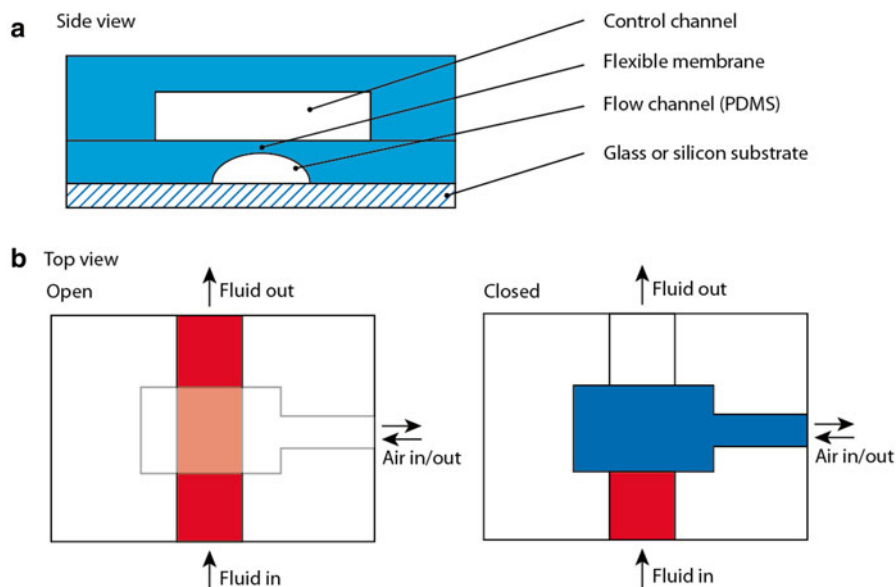
The genetic information held by the genome is transcribed into RNA either as coding RNA such as messenger RNA (mRNA), or as non-coding RNAs such as transfer RNA (tRNA), ribosomal RNA (rRNA) and the regulatory RNAs (miRNA, siRNA and other types) [50]. The set of all the RNA molecules is henceforth termed transcriptome. The noncoding RNAs play direct roles in the cellular functions in e.g. constituting the core elements of protein synthesis, regulating gene expression or protection against viral infection. In contrast, the coding RNA, such as mRNAs in most cases are categorized as mediator molecules without direct cellular effect of their own. Rather, mRNAs convey the genetic information from DNA to the ribosome, where they are then translated into a polymer of amino acids, or a protein, as stated in the central dogma of molecular biology. The transcriptome in a cell, regardless of their functions, is extensively regulated by the cellular microenvironments and the request of the cells. Therefore, probing the transcriptome would provide important information about the cell. But why is “single cell” transcription analysis necessary? It has been reported that the cells encode a subtle set of analogue parameters to modulate the responses to environmental stimuli, e.g. Tay et al. have observed a heterogeneous activation of mouse fibroblast (3T3) cells in response to the signaling molecule tumor-necrosis factor (TNF)- $\alpha$  [51]. In this scenario, bulk measurements provide relatively limited biologically relevant information in the development of cancers, especially how individual cells respond to the changes of environment differently. Moreover, considerable cell-to-cell variation with regard to transcription is an inherent feature of cancers [52, 53]. Therefore, methods for analyzing the transcriptomic level of single cells is of critical need to bring forth new and insightful information about cancer development and the potential treatment against the diseases.

In the clinical settings, RNA based methods, such as quantitative polymerase chain reaction (qPCR), are widely used in cancer diagnosis and prognosis [54–56], where the expression level of specific genes is analyzed mainly in bulk set-ups. Hitherto, transcription analyses in the single cell manner have not yet found its way to the clinic. Nevertheless, microfluidics based single cell studies at the transcriptomic level, made possible by the commercialized system supplied by Fluidigm, have been widely adopted for scientific purposes by combining quantitative Reverse Transcriptase Polynucleotide Chain Reaction (qRT-PCR) and microfluidics in cancer characterization [57–63]. The commercialized single cell qRT-PCR platform is based on the prototype reported by Quake’s group (Fig. 5.5) [64]. The basic principle is to trap single cells sorted by Fluorescence-Activated Cell Sorting (FACS) in



**Fig. 5.5** Single-cell mRNA isolation and cDNA synthesis: (a) Overview of the device, which implements five steps, including cell capture, cell lysis, mRNA purification, cDNA synthesis, cDNA purification, in one integrated device. Flow channels and control channels are depicted in green and blue, respectively. The red unrounded (rectangular profile) flow channels are where designed for affinity column construction. White boxed regions are zoomed in (b) and (c), individually. (b) The lysis ring and an NIH/3T3 cell captured in the ring. (c) The affinity column construction area and a stacked column. Scale bars are 400  $\mu\text{m}$ . Figure adapted from Ref. 62 with permission (Color online)

confined wells, and perform qRT-PCR reactions in the individual wells. The tiny nanoliter wells are generated by a fluidic circuit composed of a fluidic layer and a valve layer. These are separated by a thin elastomeric rubber member, as shown in Fig. 5.6. When pressurized, gas is applied to the valve layer, the membrane deflects and interrupts the flow in the fluidic layer [65]. As a result, trapped single cells may be analyzed within the individualized well without cross-contamination. Using this platform, Dalerba and colleagues have shown that human colon cancer tissues contain distinct cell populations of which the transcriptional identities mirror those of the different cellular lineages of the normal colon tissue [57]. In their work, more than 230 genes were evaluated in 336 single cells of three different colon epithelial cells and cancer cell lineages. Other reports have used a similar approach to investigate transcription in breast cancers [58, 59] and leukemia [60]. As an alternative to the commercial qRT-PCR platform, White and colleagues have developed a fully integrated microfluidic qRT-PCR device that implements all steps, including cell entrapment, lysis, reverse transcription and qPCR analysis in one single device, which produces a throughput of 300 cells per run [66]. The device is capable of



**Fig. 5.6** Valve control in microfluidics: Monolithic valves in microfluidic devices are typically produced by soft-lithography techniques using polydimethylsiloxane (PDMS). Shown here is a typical two-layer PDMS “push-down” microfluidic valve. **(a)** *Side view*: A thin elastomeric membrane is placed in between the flow and control channels. **(b)** *Top view*: When the control channel is pressurized, the thin membrane would be “pushed” downward and close the flow in the flow channel. The flow channel is typically positioned orthogonal to the control channel

measuring RNA levels, as well as performing single nucleotide variant detection of single cells in a high throughput manner.

In contrast to the techniques mentioned above, capable of handling many cells simultaneously but only allowing a limited number of RNAs per cell (up to 96 RNAs) to be analyzed, Ramsköld et al. have recently presented a non-microfluidic based whole transcriptomic analysis of 12 single human CTCs using mRNA-sequencing [67]. The mRNA-sequencing study was performed on fewer cells compared to the microfluidic based qRT-PCR studies (12 cells in Ramsköld et al. [67] compared to >500 by qRT-PCR in Dalerba et al. [57]). In future studies, incorporation of microfluidics with mRNA-sequencing is expected to improve the throughput of transcriptional analyses of single cells.

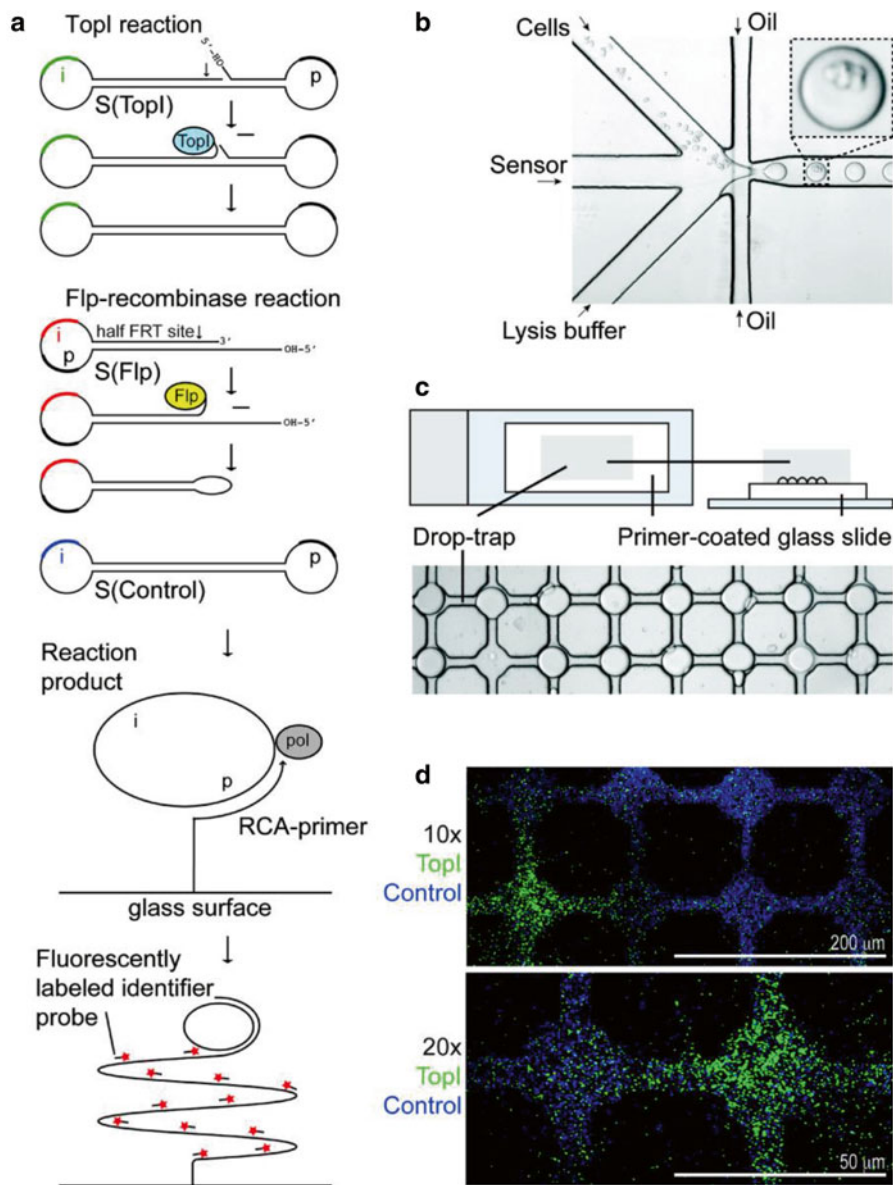
### 5.3.3 Single Cell Protein Analysis for Cancer Characterization

The human genome codes for ~25,000 genes that are transcribed and translated into proteins [68]. The process of mRNA splicing makes the number of different proteins expressed by the cell many fold higher than the number of genes [69].

These proteins, which include active enzymes, are the main determinants for the cellular processes. Many currently used drugs in cancer therapy function by targeting specific enzymatic reactions in the cell [70–73]. Consistently, changes in the activity of the target enzyme may result in elevated chemo-resistance. Since the activity of enzymes are often modified on the post-translational level, such changes may not always be recognized at the genomic or transcriptional level [74], posing a need for analysis at the post-translational protein level.

Various methods have been applied for analysis of proteins at the single cell level, including mass spectrometry, enzyme-linked immunosorbent assay (ELISA), enzymatic detection or optical approaches [75–80]. Such analyses can involve measurement of the “expressed protein amounts” or “protein activity”. For example, Qihui et al. have proposed a microfluidic device that can isolate 0–5 cells in a 2 nL volume chamber and assay up to 11 different proteins per chamber on the chip using immunostaining procedures [78]. Regarding the throughput, the microfluidic device is able to isolate and assay 100 single cells per chip.

The strategy of detecting protein amount by immunohistochemistry is well established and antibodies can be designed to recognize almost all proteins and even specific features of a protein such as posttranslational modifications (e.g. phosphorylation and other modifications) [81–84]. However, in most cases it is the function of the proteins, such as the catalytic activity for enzymes, and not the amount per se, that determines the effect of the given protein on cellular conditions, such as health, drug response etc. From that perspective, our group has developed an array of DNA nanosensors that measure cancer relevant enzymatic activities, such as human topoisomerase I (hTopI) [85–87], topoisomerase II (hTopII) [88], tyrosyl-DNA phosphodiesterase 1 (Tdp1) [89, 90], which are emerging targets for anticancer therapy. The major working principle, termed rolling circle enhanced enzyme activity detection (REEAD), utilizes the catalytic activity of the target enzyme to generate an intrinsic amplification. Take the detection of hTopI activity for example (Fig. 5.7a), the DNA nanosensors are designed as a linear DNA substrate which fold upon itself, forming a dumbbell shape. hTopI is able to recognize the substrate, cleave from the 3' end and religate the 5' hydroxyl group, turning the linear DNA into a circularized one. Subsequent process is designed to differentiate the circles from the linear pieces by isothermal rolling circle amplification (RCA). As a result, the measured fluorescence represents the individual cleavage-religation event generated by active hTopI. Perhaps the best example demonstrating the potential of combining the microfluidics with nanotechnology for single cell analysis, water-in-oil droplets generated by a flow-focusing type of droplet generator is introduced to encapsulate individual cells along with the above-mentioned DNA nanosensors. The droplets provide a confined environment for the serial of biochemical reactions, enabling the enzymatic activities to be observed at the single cell level.



**Fig. 5.7** Combining DNA nanosensors and microfluidics for single cell based analysis of enzymatic activities: **(a)** DNA substrates S(TopI) and S(Flp) are oligonucleotides that target cleavage-ligation by human topoisomerase I (TopI) and Flp, respectively. The detection, termed rolling circle enhanced enzyme activity detection (REEAD), initiates by recognition of enzymes to the substrates, which results in a circularized product. Subsequently, the circles allow a solid-support rolling circle amplification (RCA), which generate  $\sim 10^3$  tandem repeat of amplified products. The results are visualized by fluorescence microscopy at the single-molecule level by hybridization of fluorescently labeled probes. **(b)** The droplet microfluidics is introduced to encapsulate individual cells along with DNA substrates and lysis buffer, in picoliters of water-in-oil droplets. **(c)** The droplets containing circularized DNA are then confined in a drop-trap on a primer-coated glass slide, where the RCA takes place. **(d)** The observed enzymatic activities from single cells are visualized as fluorescence signals: *green* (TopI) and *blue* (control). Figure adapted from Ref. 78 with permission (Color online)



## 5.4 Conclusion

Microfluidics-based approaches have been promoted for many biochemical applications, such as drug screening [91], nucleic acid amplification [92], and analysis of chemical reactions [93]. Therefore, it becomes a natural extension to take advantage of microfluidics for single cells interrogation. The device can be tailored to exploit physical and/or biological differences for isolation of particular cell types from a population of cells, such as enrichment of CTCs, and separation of CSCs from non-stem cells. The small dimensions of microfluidic devices have also enabled many unique features, such as gentle capture of live or rare cells [34], which avoids possible interferences for the subsequent analysis. Microfluidics also presents an opportunity to integrate many functions, such as isolation, biochemical reaction and detection, in a single device. For example, recent evidence has shown a high degree of heterogeneity even within a population of CTCs [94]. Therefore, combining CTC enrichment and molecular analysis at the single cell level is clinically important for the characterization of rare cell phenotypes, including CTCs in various stages during the cancer progression or perhaps study of how CSCs behave differently compared to non-stem cells. Furthermore, the isolated cells can be directed to next-stage analysis on-chip (e.g., genomic, transcriptomic or proteomic analysis), or on-chip cell culture as part of the analysis. Such integration will speed up the cancer characterization process while eliminating the intermediate sample transfer procedures typically required in macro-scale approaches.

Furthermore, advancement in cellular, microscopic, or nanoscaled molecular techniques is pivotal to the single cell analysis. The development of reliable biomarkers shall closely follow, if not precede, the emergence of microfluidics. For this purpose, immunostaining remains the prevailing technique for cellular or protein recognition. Recent progress in nanotechnology has joined the league by providing immunomodulatory agents engineered with nanostructure materials, including metallic nanoparticles, quantum dots, or nanotubes, to either improve the efficiency immunorecognition or enhance the detection sensitivity [95]. On the other hand, nanoparticles possessing unique photophysical properties, such as semiconductor nanocrystals and noble metal nanoclusters, also serve as unique fluorescence analogs in illuminating various forms of biological analytes through different signal transduction pathways. Taken together, the latest development of both nanotechnology and microfluidics is expected to encourage new excitement in the field of single cell analysis.

Despite the large variety of available approaches targeting and analyzing single cells, we are still in the infancy to uncover the implications of cellular heterogeneity. Many challenges have yet to come. One of the foremost, existing available approaches for single cell analysis is still expensive and technically complex, in particular those require high precision pumps or delicate valving control, which prevent the adaptation in the clinical studies. Future development of microfluidics-based single cell analysis is expected to reduce the production cost, sample consumption and to avoid human operational error by integration of sample preparation and subsequent data acquisition onto one single device. However, tradeoff of microfluidics remains on the



analysis speed, especially when it comes to large sample volume. The capability of parallel processing, when made possible, will hopefully accelerate the adoption of microfluidics for large-scale single cell analysis. Accessible single cell analysis, however, requires collective efforts including further engineering optimization of microfluidic systems and suitable nanoscaled molecular analysis approaches on the chip. The new possibility to reveal the characteristics of rare cells is expected to ultimately lead to clinical implications in the combat of cancers.

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