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REVIEW ARTICLE

The future of microfluidics in immune checkpoint blockade

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Abstract

Recent advances in microfluidic techniques have enabled researchers to study sensitivities to immune checkpoint therapy, to determine patients' response to particular antibody treatment. Utilization of this technology is helpful in antibody discovery and in the design of personalized medicine. A variety of microfluidic approaches can provide several functions in processes such as immunologic, genomic, and/or transcriptomic analysis with the aim of improving the efficacy and coverage of immunotherapy, particularly immune checkpoint blockade (ICB). To achieve this requires researchers to overcome the challenges in the current state of the technology. This review looks into the advancements in microfluidic technologies applied to researches on immune checkpoint blockade treatment and its potential shift from proof-of-principle stage to clinical application.

Introduction

Cancer immunotherapy (CIT) is a promising anticancer treatment strategy with more efficient survival rate and lesser adverse effect than conventional treatments such as surgery, radiotherapy, chemotherapy, and molecular targeted therapy [1]. The increasing demand for immunotherapeutics in clinical research as well as in hospital and clinics has fueled a market growth with a USD 242.86 billion forecast by 2026 [2]. The immune system has the ability to recognize and kill cancer cells without toxicity to normal and healthy ones [3]. This treatment approach dates back to the late 1800s but immunotherapy's major breakthroughs in understanding the mechanisms in full T-cell activation and tumor-induced immunosuppression came about in the last decade [1, 4]. At present, immunotherapy is an established strategy and has become a new standard of care in clinical treatments with unprecedented survival improvements to patients with advanced-stage tumors [5, 6].

Although significant responses have been recorded, not all patients benefit from the treatment approach because of the immune system's diversity and dynamic nature, together with inter- and intratumor heterogeneity. Tumors can adapt, mutate, and escape immune surveillance [3]. The efficacy of immunotherapeutics in modulating cancer progression is still lacking for most human cancers [4]. The small number of success of CIT agents poses a challenge to clinicians and researchers [7–9]. For instance, only 12.46% of US patients were reported in 2018 to respond to checkpoint inhibitor drugs [10]. Overall, about 20–40% of cancer patients were found to respond to immunotherapy [11]. To further improve the treatment effectiveness and patient response rates, more specific biomarkers and immune checkpoint inhibitors are needed to be identified. This would require better predictive tools and assays for careful screening of responders to immunotherapy [1].

Microfluidic platforms have the capacity to address the needs and challenges facing CIT. These tools are capable of single-cell analysis, mimic tumor microenvironment (TME), and perform real-time measurement. Microfluidic technology provides high precision of liquid manipulation that conventional bench-top approaches cannot perform [12, 13]. Among other advantages include reduced size of operating systems, reagent consumption, waste production, and power requirement, while at the same time having more speed in analysis and flexibility in design. For example, a

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microfluidic platform for exosome analysis can increase the assay sensitivity and reduce assay time and sample requirement by at least two orders of magnitude [14]. Conventional bulk methods, meanwhile, are limited in terms of depth and dimensionality of data, as it does not take into account the phenotypic and functional states of heterogenous cells [3, 15]. On the other hand, microfluidics technology for single-cell analysis has the ability to address complex immunological issues. Thus, it is capable of identifying rare cell subsets and resolution between cells with identical expression patterns.

A variety of microfluidic approaches can provide several functions in processes such as immunologic, genomic, and/or transcriptomic analysis with the aim of improving the efficacy and coverage of immunotherapy. These devices have shown great promise in profiling TME, identifying biomarkers in biopsies and revealing the biomarkers' role in triggering or arresting antitumor immune response. Advancements in microfluidic techniques have also enabled researches to study sensitivities to immune checkpoint blockade (ICB) and help clinicians design personalized medicine [12].

In this review, we highlight immune checkpoint blockade in CIT and particularly discuss various microfluidic platforms, both clinically approved and proof-of-concept devices, and their applications in disease monitoring. We look into the platforms' capability in single-cell profiling and small conditional RNA (scRNA) sequencing. We also talk about microfluidic technologies developed to discover and evaluate immune checkpoint inhibitors, test the efficacy of immune checkpoint blockade therapies, and monitor treatment response. Finally, we discuss the future outlook of microfluidic technology applications in immune checkpoint blockade and its potential shift from proof-of-principle stage to clinical application.

Immunotherapy and the TME

CIT is a promising cancer treatment strategy that utilizes the body's ability to launch an immune response, primarily targeting the immune system or TME [16]. At present, immunotherapy is an established strategy and has become a new standard of care in clinical treatments with unprecedented survival improvements to patients with advanced stage tumors [5, 6]. For example, significant results have been reported on metastasis reduction in melanoma and kidney cancer patients under immunotherapies. Complete response was also achieved with the use of high-dose interleukin (IL)-2 [7, 17]. Use of CTLA-4 antibody-based drug has also been reported to clear 22% of patients with advanced late-stage melanoma [18].

Although significant responses have been recorded, not all patients benefit from the treatment approach because of the immune system's diversity and dynamic nature together with inter- and intratumor heterogeneity. These limitations lead to the use of combination strategies and quest for biomarkers and receptor routes that can destroy a wide range of cancers in all patients [5, 6, 19]. Novel biomarkers including specific mutations and immune function markers, together with monitoring and evaluation of the TME through new strategies, can improve early diagnosis and therapeutic efficacy prediction that are necessary to select the patients who can get the therapeutic benefit.

Targeting or modulating the TME has caught the attention of clinicians, as this microenvironment plays an important role in suppressing antitumor immunity with its significant heterogeneity [16]. This cellular niche, which is made up of tumor cells, fibroblasts, tumor-infiltrating immune cells, signaling molecules, cancer-associated fibroblasts, tumor vasculature, and extracellular matrix, takes part in tumor development, progression, and recurrence. This makes profiling TME an important topic for research in regulating tumor response for improved immunotherapy efficacy for multiple types of cancers [16, 20].

There are several therapeutic strategies being used and/or explored to date. Some Immunotherapies block inhibitory pathways and immunosuppressive cells in the TME to intensify antitumor immunity. This is done with the use of monoclonal antibodies (mAbs) and checkpoint inhibitors (i.e., anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), anti-programmed cell death protein 1 (PD-1), and anti-programmed death ligand 1 (PD-L1)), as well as genetically modified immune cells via adoptive cell transfer (i.e., chimeric antigen receptor, T-cell receptor engineered T cells). Other therapies kill cancer cells by boosting the immune system using cytokines (i.e., IL-2, Janus kinase inhibitors), cancer vaccines (i.e., cancer cell antigen, peptide vaccines, and genetic or viral vaccines), or a combination of therapies [3].

Even though immunotherapy strategies have delivered unprecedented responses from patients with advanced-stage tumors, the problem with limited clinical response persists. The small number of success of CIT agents poses a challenge to clinicians and researchers [7–9]. For example, immune checkpoint antagonists targeting the PD-1 pathway has a response rate of about 20–30% across tumor types. Positive objective response rates (ORRs) from patients under immune checkpoint blockade treatment were reported on malignancies, 20% on gastric cancer, 12–25% on head and neck squamous cell carcinoma, 20% on ovarian cancer, 15% on small-cell lung cancer, 20% on triple-negative breast cancer, 25% on urothelial carcinoma, and 65–85% on Hodgkin's lymphoma [7, 21, 22]. Combination of

CTLA-4- and PD-1-blocking antibodies resulted in 40% ORR across all doses [11].

Monitoring cell heterogeneity

To have a more potent therapy and achieve complete response, it is necessary to study the immune cell components, its heterogeneity, and interaction with cancer in arresting or promoting the latter's progression, so as to understand the immune effector mechanisms. Conventional technologies that uses bulk samples are limited in terms of depth and dimensionality of data. This is because bulk analysis of immune cell populations only gives information on the average immune response without taking into account the phenotypic and functional states of these heterogenous cells and its intercellular signaling with other cell subsets [3, 15]. This means that identification of rare cell types and resolution between cells with identical expression patterns are not covered [15].

Monitoring the behavior of each immune cells has resulted to discoveries of several biological functions that were not observed in bulk analysis. This single-cell analysis provides information on genotype, phenotype, protein secretion, proliferation, maturation, activation, signaling pathways of each activated cell, and the intercellular communication among different immune cells [3, 13]. For example, profiling individual tumor cells can provide insights into its cellular behavior, which could result to identification of more potent drugs and improve the response rates of patients to therapies [23].

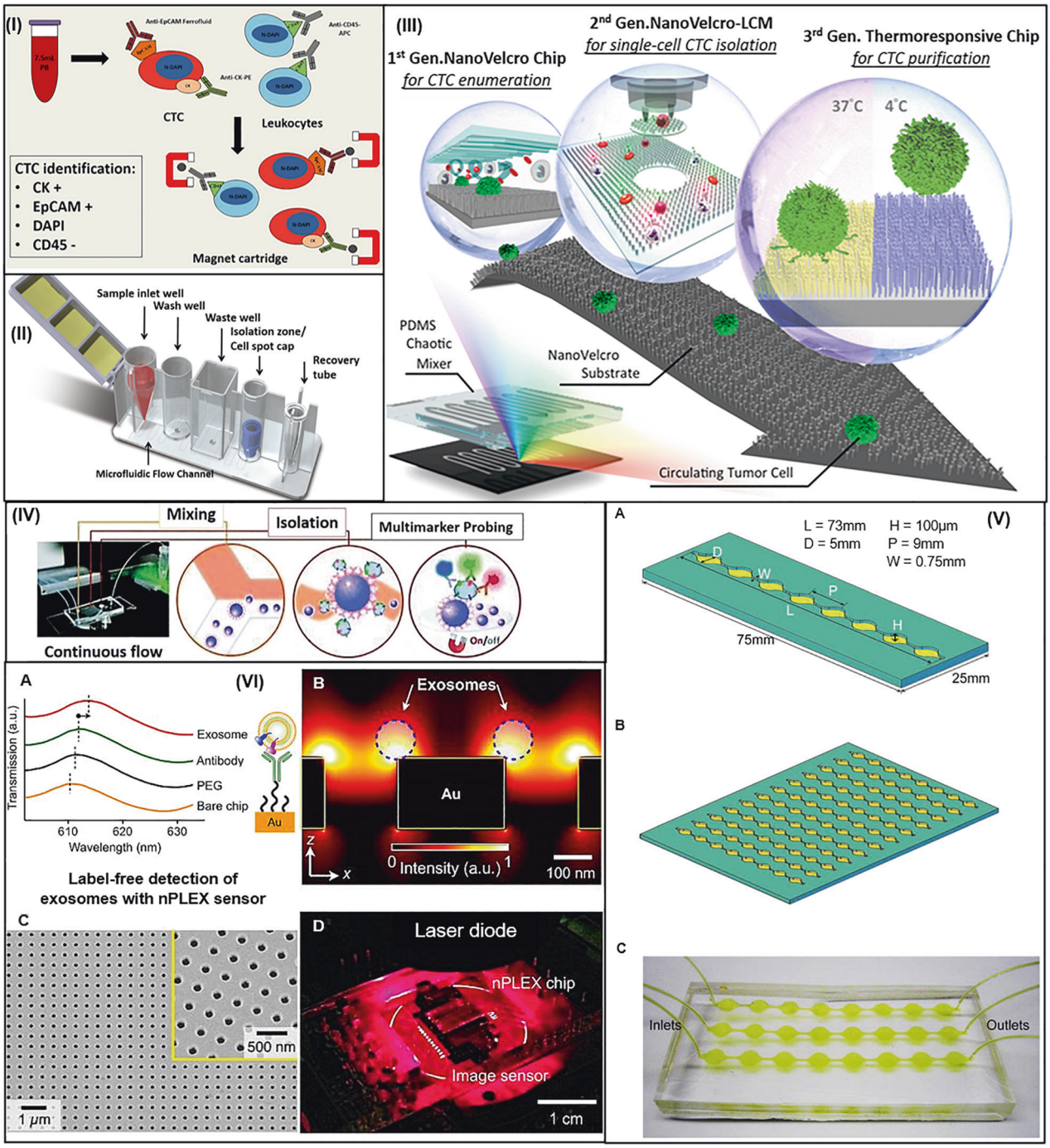
At present, many researches are being conducted on single-cell analysis with focus on technology development to provide new tools for novel immunotherapies, point-of-care diagnostics, cell profiling and drug screening. These approaches address complex immunological issues that conventional bulk methods fail to address. The design and development of point-of-care devices, serving as alternative clinical tools, allows for monitoring and profiling of complex heterogenous cellular and molecular activities with the aim of improving response prediction and treatment optimization [13, 24].

Microfluidic technology

Among these technologies, the use of microfluidic platforms is popular. For the past several years, it has shown advantages by providing miniaturized devices with fast isolation speeds and high efficiency that allows acquisition of more information from single cells at higher resolution. It can be integrated into multiple workflows, which allows parallelization and streamlining of complex protocols with minimal sample and reagent volume requirement. Microfluidic technology provides high precision of liquid manipulation that conventional bench-top approaches cannot perform [12, 13]. Automation and control of analytical functions, manipulation of cells, its environment and intercellular activity, mapping distinct immune cell subsets, as well as simultaneous detection and quantification of a number of secreted molecules (multiplexing) are possible in the design of these platforms [13, 22].

For instance, the microfluidic platform developed by Merouane et al. [25] called time-lapse imaging microscopy in nanowell grids uses sub-nanoliter wells and live-cell microscopy to dynamically monitor cell-to-cell interactions between immune effector cells and tumors cells, as well as its cytotoxicity, cell motility, and cell survival. Using fluorescent labeled human T cells, natural killer (NK) cells and target cells (NALM6, K562, and EL4), the said platform was able to do automated quantification of cell location, morphology, movement, and death without manual proofreading [22, 25]. Such high-throughput operation is advantageous over conventional techniques, which handles 10–100 representative cell pairs, as many biologically significant cellular subpopulations and biotechnologically relevant protein secreting cells are rare.

Another microfluidic platform used for monitoring of single-cell pair activity and protein secretion is the single-cell barcoding chip (SCBC) that was developed by the Heath group. SCBC contains arrays of microwells and sets of immobilized barcode-like patterned capture antibody for proteins and other detections. This chip is capable of multiple protein quantification from the same cell using fluorescent sandwich immunoassay readout [26, 27]. Requiring only a small amount of sample, the SCBC can accommodate up to around 40 plex protein detection from a single-cell [22]. A similar concept is the beads-on-barcode antibody microarray (BOBarray) by Yang et al. [22, 28] and the work of Armbrecht et al. [23] on single-cell protein profiling with barcode beads. In these works, fluorescently barcoded beads are either immobilized on glass surface and positioned on top of microwell (BOBarray) or co-captured in a microwell, together with the cell of interest, using a magnet. The BOBarray made use of different bead sizes and colors to encode 12 different proteins and characterize the organ-of-origin and drug targets of rare tumor cells isolated from blood samples. Meanwhile, the platform of Armbrecht et al. [23] was able to perform on-chip immunoassay and multiplexed single-cell protein quantification of three mammalian cell lines (MCF-7, HEK-293T, and SK-BR-3 cells). In using the barcoded beads, the limits on flexibility, processing time, and occurrence of background signal when unprocessed are avoided.



Applications in therapy assessment and disease monitoring

Currently, there are a number of fluidic devices that have been developed and/or clinically approved for pre-screening, diagnostic, prognostic, therapy assessment, and monitoring of the disease. One of the Food and Drug Administration (FDA)-approved fluidic technologies is CellSearch (Menarini-Silicon Biosystems, Bologna, Italy), shown in Fig. 1-I. A gold standard for circulating tumor cell (CTC) enrichment, this device detects and quantifies CTCs of epithelial origin (CD45−, EpCAM+, and cytokeratins 8, 18+, and/or 19+) in the whole blood of metastatic breasts, prostate, and colorectal cancer patients. Using antibody coated magnetic particles to target epithelial cell adhesion molecule (EpCAM), the amount of detected CTC, against a predetermined threshold, can be used to predict prognosis and assess progression-free survival and overall survival

◀ **Fig. 1 Microfluidic devices developed for therapy assessment and disease monitoring. I** Schematic diagram of the CellSearch system technology for detection and enumeration of CTCs from peripheral blood. Magnetic iron nanoparticles coated with anti-EpCAM antibody are used to give magnetic properties to epithelial cells. Anti-CK and anti-CD45 fluorescent antibodies are used to discriminate target cells. CTC are separated using a strong magnetic field and counted by digital fluorescent microscopy [Reproduced from ref. [30] with permission from the *Frontiers in Oncology*, Frontiers Research Foundation]. **II** Illustration of the IsoFlux device for CTC isolation, showing its parts and flow path [Reprinted from ref. [34], Copyright (2013), with permission from Elsevier]. **III** Diagram of the NanoVelcro CTC chip showing the features and improvements through four generations [Reprinted from ref. [36], Copyright (2018), with permission from Elsevier]. **IV** Image of ExoSearch and illustration of its workflow: mixing, isolation, and in situ, multiplexed detection of circulating exosomes [Reproduced from Ref. [32] with permission from the *Royal Society of Chemistry*]. **V** ExoChip design and features (A). Illustration of single channel and its dimensions (B). A twelve-channel chip for simultaneous analysis of multiple samples (C). Image of the polydimethylsiloxane (PDMS)-based ExoChip prototype showing the flow of serum for exosomes capture [Republished with permission of *Royal Society of Chemistry*, from ref. [36]; permission conveyed through Copyright Clearance Center, Inc.]. **VI** nPLEX technology for label-free detection of exosomes (A). A graph illustrating the changes in transmission spectra showing exosome detection with nPLEX. Antibody conjugation and specific exosome binding were monitored by transmission spectral shifts (B). Enhanced electromagnetic fields tightly confined near a periodic nanohole surface shown as finite-difference time-domain simulation (C). SEM image of the periodic nanoholes (diameter = 200 nm, periodicity = 450 nm) in the nPLEX sensor (D). Image of nPLEX imaging system [Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, *Nature Biotechnology* (ref. [41], Copyright (2014)].

[12, 29, 30]. Janning et al. [31] and Nicolazzo et al. [32], e.g., have used CellSearch in investigating the role of PD-L1$^+$ CTCs in the clinical setting. The group of Janning compared EpCAM-based (CellSearch®) system to epitope-independent method (ParsortixTM system) in assessing PD-L1 expression of CTCs from non-small-cell lung carcinoma (NSCLC) patients. Meanwhile, Nicolazzo et al. [32] used CellSearch in analyzing CTCs from NSCLC patient treated with Nivolumab [31–33].

Another microfluidic device for CTC enrichment is the IsoFlux system (Fluxion Biosciences, Alameda, California, USA), shown in Fig. 1-II. Combining antibody-based magnetic bead separations with flow cytometry precision, Isoflux claims to have higher sensitivity in CTC identification from carcinoma patients as compared to CellSearch [12, 34]. For instance, Isoflux enrichment system and CTC isolation platform were used by Po et. [35] to aid their study on screening PD-L1 levels induced from liquid biopsies (melanoma CTCs), though targeted CTC isolation and specific immunostaining-based CTC identification combined with PD-L1 detection.

Similarly, NanoVelcro (Fig. 1-III) is a microfluidic chip for CTC identification, capture, and release. The device, however, makes use of nanowires coated with anti-EpCAM antibody where the tumor cells stick on. Once captured, cytokeratin/ CD45/4′,6-diamidino-2-phenylindole staining can be performed to determine putative CTCs. The Nano-Velcro device allows the analysis of tumor specific RNA markers by purifying CTC without damaging RNA transcripts [12, 36]. Antibody-based Nanovelcro microfluidic chip have been used by Winograd et al. [37] to evaluate the feasibility of isolating and detecting CTC-expressing PD-L1 in patients with HCC. With the aid of the multi-marker antibody-based CTC capture device, they evaluated the PD-L1 expression in HCC CTCs and its association with HCC tumor stage, and the prognostic impact and predictive ability of PD-L1$^+$ CTCs to immunotherapy treatment response [37].

Other than CTC, immune-affinity microfluidic devices have been applied to capture exosomes in liquid biopsy, as it is believed to promote remodeling of distant, metastasis organs. One of these is the ExoSearch chip (Fig. 1-IV), which is capable of capturing blood plasma exosomes and perform multi-marker probing for ovarian cancer. The microfluidic method allows enriched blood plasma exosome preparation for in situ, multiplexed detection and measurement of exosomal tumor markers (CA-125, EpCAM, CD24) using immunomagnetic beads [12, 38]. Likewise, ExoChip (functionalized with antibodies against CD63) is capable of on-chip isolation, detection, quantification, as well as recovery of exosomes with intact RNA for exosomal-microRNA profiling via openarray analysis (Fig. 1-V) [39]. nPLEX- nano-plasmonic exosome sensor (functionalized CD24, CD63, and EpCAM) can perform simultaneous isolation and surface plasmon resonance (SPR)-based multiplexed detection of ovarian cancer-derived EpCAM (+) and CD24 (+) relative to CD63(+) exosomes (Fig. 1-VI) [40, 41].

Capture and measurement of exosomes or exosomal tumor markers (i.e., exosomal PD-L1) using these microfluidic platforms would be beneficial in identifying response to therapeutics that block exosome production. For instance, exosomes derived from lung cancer cells that expresses PD-L1 has a role in immune escape and tumor growth promotion [42]. This is because exosomes have the ability to impair immune functions through cytokine production reduction and CD8+ T-cell apoptosis induction [43]. Thus, use of this technology and clinical application would provide useful insight in cancer management and immune checkpoint blockade, in particular.

Microfluidics and scRNA sequencing

One of the great challenges in cancer therapy is the intratumoral heterogeneity, which allows cancer evolution and therapeutics evasion. Analysis of CTCs in blood, e.g., has the potential to use DNA as clinical diagnostic marker.

In these cases, scRNA-seq can be applied to discover unknown tumor features and generate cancer evolution models through assessment of transcriptional heterogeneity and analysis of expression profiles for specific pathways. With these means, coding mutations and fusion genes can be discovered from CTCs and other phenotypic consequences of DNA and RNA variants [44]. Therefore, CTCs are important noninvasive source of clinical information, which can provide access to complete transcriptome and proteome, as well as insights into biomarker characterization, early cancer diagnostics and cancer development evaluation, and therapeutic efficacy prognosis [45, 46]. In contrast, conventional biopsy is invasive, poses a challenge in accessing tumor sites (i.e., inside lungs or brain), and may have negative impact on patient compliance [46]. The development of microfluidic platforms, meanwhile, provides a noninvasive means for CTC isolation. It is more convenient, accurate, and harmless to patients [45]. Integration of multistep isolation procedures and advanced nanotechnologies can be done for higher-performance of CTC isolation.

However, scRNA-seq has some limitations, such as low capture efficiency and high dropouts, which misses weakly expressed genes. The data produced are also nosier and more variable in comparison to bulk RNA sequencing (RNA-seq). Improving the RNA capture efficiency and transcript coverage could minimize the noise in scRNA-seq protocols [47].

Advances in microfluidics technology has enabled integration of scRNA-seg in its platforms. Single-cell isolation platforms have attracted researchers because of its low sample consumption and analysis cost, as well as its precise fluid control, which allows single-cell gene expression profiles to be quantified in a highly parallel manner [44]. Together with the developments in cellular barcoding, throughput of scRNA-seq has significantly increased with microfluidics integration [47]. This throughput increase was achieved with the improvements in cell-capture techniques like in droplet microfluidics.

For example, droplet microfluidics was employed by Demaree et al. [48] for a high-throughput and low -deviation single-cell sequencing (SiC-seq) platform (Fig. 2-I), which is capable of separation, amplification, and barcoding of the single-cell genome [49]. Another high-throughput scRNA-seq platform is the Microwell-seq (Fig. 2-II) by Han et al. [50]. The said platform improved the detection abundance at a lower cost as compared to oil droplet techniques [49, 50]. Meanwhile, Habib et al. [51] developed a droplet microfluidic platform (DroNc-seq) integrated with single-cell nuclear RNA-seq, which provides a low-cost, high-sensitive, and high-efficiency cell classification (Fig. 2-III) [49].

Future microfluidic platforms should also be capable of not only CTC isolation but also ctDNA and exosome enrichment, as it enables complementary approaches to cancer heterogeneity understanding and development of personalized treatments. For example, measurement of ctDNA and exosome levels could be used as predictive biomarker in patients under ICB treatment [52]. The advancements in microfluidic technologies mentioned above can provide automated and high-throughput clinical validation needed for a more effective personalized medicine.

Role of microfluidics in immune checkpoint blockade

Immune checkpoint blockade

The discovery of the underlying mechanisms that prevents the immune system from attacking tumors by Dr. James Allison and Dr. Tasuku Honjo, 2018 Nobel Prize awardees in Physiology or medicine, has led to a cancer therapy by inhibition of negative immune regulation [3, 18]. Allison, who worked on CTLA-4, a protein that is a negative regulator of T-cell activation, discovered that blocking its inhibitory effect enhances immune response against tumor cells resulting in a significant growth reduction or even complete tumor rejection. This was demonstrated in an in vivo antibody-mediated CTLA-4 blockade using murine models. Honjo, on the other hand, discovered PD-1, a member of the immunoglobulin gene superfamily [18]. The PD-1 receptor is expressed on activated T cells, regulatory T cells, B cells, macrophages, and NK cells. Its binding to its ligand, PD-L1 or PD-L2 results in the repression of T-cell proliferation and cytokine secretion. This mechanism enables tumors to evade antigen-specific T-cell immune response [8]. Therefore, blocking these proteins with mAbs or immune checkpoint inhibitors can enhance antitumor activity of T cells, normalize TME, and prevent hematogenous spread of tumor cells [7, 16, 51]. Clinical trials have shown the potency of this treatment strategy to patients with different types of metastatic cancers and have led to approval of therapeutic drugs by FDA for use in numerous cancer types, consequently confirming the immunotherapy-targeted TME.

Recent studies on immune checkpoint blockade are investigating on antibody-based therapies that target multiple inhibitory receptors and other T-cell inhibitory receptors such as T-cell immunoglobulin domain and mucin domain-3, lymphocyte-activation gene 3 (LAG-3), V-domain immunoglobulin suppressor of T-cell activation, and B- and T-lymphocyte attenuator [53, 54].

Microfluidics in ICB treatment

From here on, this review will focus on microfluidic technologies applied to immune checkpoint blockade treatment

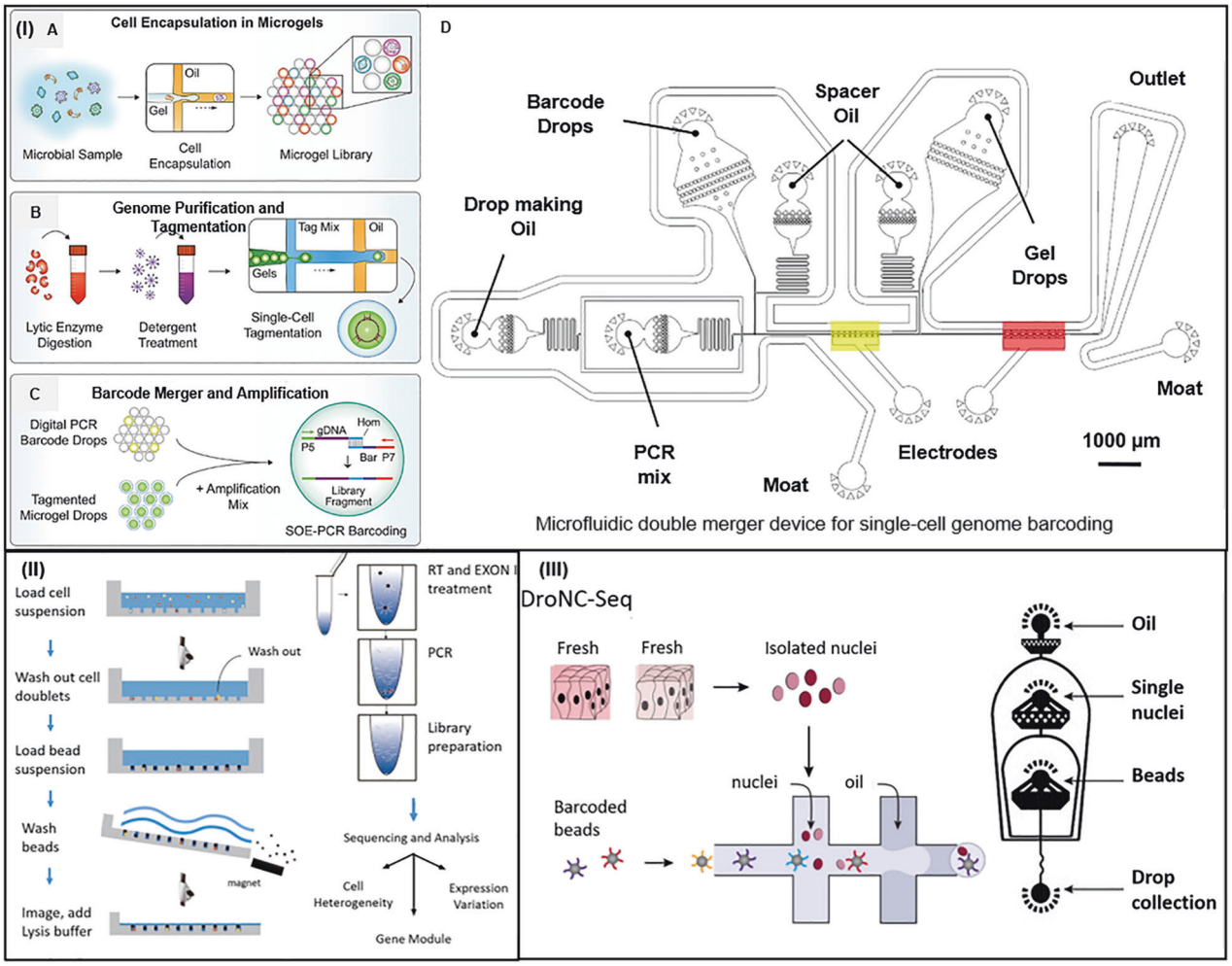


Fig. 2 Microfluidic tools with integration of single-cell RNA sequencings and analysis. I Single-cell genome sequencing (SiC-seq) is a high-throughput microfluidic platform that uses droplets to isolate, amplify, and barcode the genomes of single cells (A). Illustration of single-cell encapsulation in microgels via a dropmaker device (B). Workflow for bacterial genomic DNA purification and microgel tagmentation (C). Merging a digital PCR barcode, a tagmented microgel genome, and an amplification mix at a rate >1 kHz. The SOE-PCR splices a unique single-cell barcode onto the tagmented genome and selectively amplifies fully barcoded constructs off-chip [Adapted from ref. [48]]. **II** Illustration of the basic workflow for Microwell-seq. [Reprinted from ref. [50], Copyright (2018), with permission from Elsevier]. **III** Overview of DroNc-seq and schematic illustration of the microfluidic device [Reprinted by permission from Springer Nature Customer Service Centre GmbH]: ref. [51], Copyright (2017)].

strategies and how these technologies are helping to shape the future of the immune-treatment approach.

The use of microchannels and chambers in microfluidic devices enable the precise control of flow and cell distribution, which can establish physical (i.e., interstitial pressure) or chemical (i.e., cytokines) gradients. This make it a powerful tool to study longitudinal monitoring of tumor cell interactions by mimicking metastatic environment [17]. Replicating these environments in vitro, using patient-derived cells, can provide information on targeted therapeutic approaches [55]. Here, microfluidic assays can be designed to test the efficacy of immune checkpoint blockade therapies or in combination with drugs to target immune regulatory pathways in order to shed light on the mechanisms that trigger or arrest antitumor response. At present, there are a number of studies using microfluidic tools to evaluate the sensitivity of immunotherapies, particularly on immune checkpoint blockade, as well as identify immune cell subsets responsible for the antitumor response [23, 55, 56].

Many researches are focused on the discovery and/or evaluation of anti-PD-1 antibodies and the corresponding immunotherapy. For example, Adler et al. [57] made use of an emulsion droplet microfluidic device to screen and discover new anti-PD-1 antibodies in wild-type mice. Here, single-cell suspension of B cells, fluorocarbon oil, and oligo-DT beads were made to flow in a co-flow emulsion droplet chip as shown in Fig. 3-I. They identified 269 molecules, expressed as yeast single-chain fragment (scFv), which bind to PD-1 protein and screened the high-affinity binders. This device presents an alternative to hybridoma-

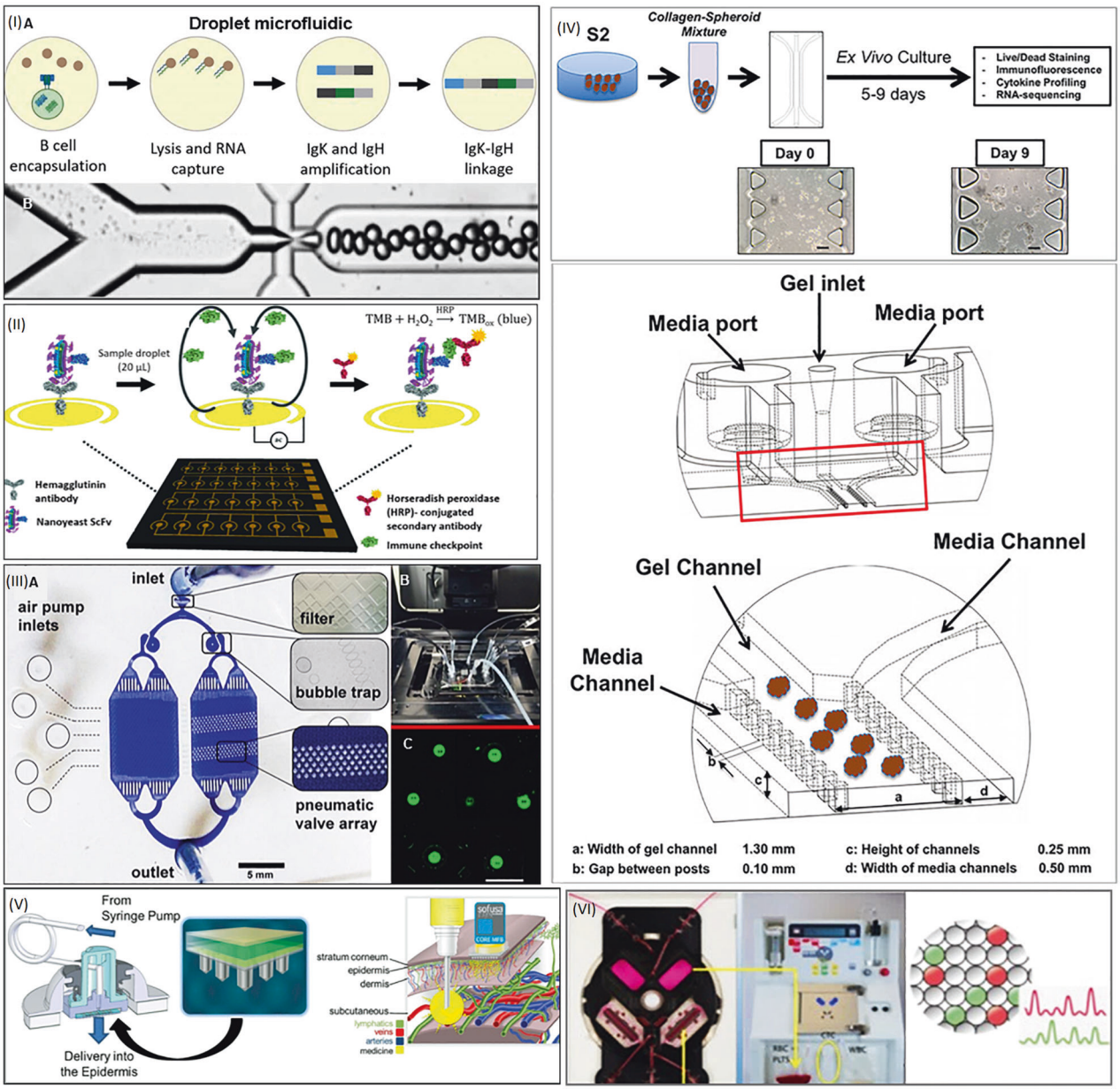


Fig. 3 Microfluidic tools for the evaluation of the efficacy and sensitivity of immunotherapies. I Droplet microfluidics platform used for scFv library generation to identify PD-1-binding molecules. (A) Workflow for B-cell encapsulation with oligo-dT beads and lysis solution for library generation. Droplets with mRNA-bound beads are purified and injected into second emulsion with an OE-RT-PCR amplification mix that generates DNA amplicons that encode scFv with native pairing of heavy and light chain Ig. (B) Image of the channel junction that generates droplets [Reproduced from ref. [47] with permission from mAbs, Taylor & Francis]. **II** Multiplexed immune checkpoint biosensor (MICB) uses electrodes functionalized with target-specific nanoyeast ScFvs for parallel detection of PD-1, PD-L1, and LAG-3 in human serum [Republished with permission of *Royal Society of Chemistry*, from ref. 58]; permission conveyed through Copyright Clearance Center, Inc.]. **III** A valve-based microfluidic device for single-cell compartmentalization and granzyme b profiling. (A) Image of the chip with parts magnified. (B) Device mounted on the microscope stage connected to fluid and air pumps. (C) Image of the chambers with fluorescence signal. [Reproduced from ref. [46] with permission from Ivyspring International]. **IV** 3D microfluidic cell culture chip to evaluate the immune-checkpoint sensitivity of MDOTS/PDOTS. (A) Chip is injected with 40–100 µm (S2) tumor fraction for ex vivo culture (scale bars: 100 µm). (B) Schematic diagram showing the center gel region with posts separating the gel region from the anti-parallel side channels [Reproduced from ref. [51] with permission from the *Royal Society of Chemistry*]. **V** SOFUSA™ Nanotopographical device (schematic) for anti-CTLA-4 infusion into sub-epidermal space to determine antitumor response of checkpoint blockade therapy [Reproduced from ref. [57] with permission from the Ivyspring International]. **VI** Microfluidic enrichment of CTCs in digital CTC assays using CTC-iChip where whole blood specimens are depleted with normal hematopoietic cells to generate highly enriched CTC population. RNA isolated from these cells is reverse transcribed into cDNA, within lipid droplets, and subjected to ddPCR, quantifying the number of transcripts for each gene [Reproduced from ref. [49] with permission from *PNAS*].

based antibody discovery but leads on comprehensiveness and turnaround time as compared to conventional well-plate screening. Although, humanizing these synthesized mAbs and subjecting to in vitro affinity maturation are needed in order to be used in anti-PD-1 therapies [57]. Wuethrich et al. [58], on the other hand, developed a multiplexed immune checkpoint biosensor, a combination of microfluidic sandwich immunoassay and in situ nanofluidic mixing that is capable of parallel detection of PD-1, PD-L1, and LAG-3 in human serum. The device is composed of 28 circular gold electrodes serving as alternating current electrohydrodynamic mixer via electric field application, which stimulates the immune checkpoint interaction with the nanoyeast ScFv functionalized electrodes (Fig. 3-II). The said device can do simultaneous analysis of up to 28 samples from a single sample drop (about 20 μL) per target immune checkpoint using high-affinity yeast cell-derived chain variables [58, 59]. In our work, we developed a microfluidic platform to measure single-cell granzyme B (GrB) expression as potential biomarker for evaluating anti-PD-1 antibody therapy response. The said platform, shown in Fig. 3-III, is integrated with pneumatic valves to compartmentalized trapped human peripheral blood mononuclear cells (PBMCs) from healthy donor and anti-PD-1 antibody-treated lung cancer patients. The expressed GrB molecules from individual cells were quantified fluorometrically through the cleaving of a peptide substrate (Ac-IEPD-AFC), which was introduced in the microchambers. Fluorescent immunostaining was also performed to distinguish specific cell subsets producing GrB. Here we observed a marked increase in GrB expression in the anti-PD-1-treated lung cancer patient sample as compared to the PBMC from healthy donor. Within the patient cell expression profile, TCR+ Ig-G4+ cells were found to have high activity, which signifies PD-1 blockade treatment response [56].

Several groups have made use of a three-dimensional (3D) microfluidic cell culture system (Fig. 3-IV) to evaluate the immune-checkpoint sensitivity of murine or patient-derived organotypic tumor spheroids (MDOTS/PDOTS). The use of this tool arises from the need of a convenient and easily accessible ex vivo diagnostic systems that can recapitulate patient-specific tumor biology for immunotherapy response prediction (i.e., response of patient tumors to ICB treatment) [60]. Conventional culture methods that model the efficacy of immunotherapies are often faced with a challenge to accurately recapitulate in vivo models. Using murine models to circumvent this challenge is time and resource intensive, not to mention the inconsistencies between murine and human immune systems. Thus, it is a poor predictor of clinical performance [61]. The use of mouse models does not also permit mechanistic studies to monitor real-time tumor progression and assay as function of local environment parameters [62, 63]. With resistance to PD-1 blockade still a challenge and biomarkers to guide treatment lacking, new model systems adaptable to mouse cancer models and human cancer can advance the field of immunotherapy. The 3D microfluidic culture systems can provide a greater level of precision in interrogating the tumor immune microenvironment and evaluating other complex biological phenomena. For the past years, there have been an increasing interests to using MDOTS/PDOTS in profiling response to PD-1 blockade to demonstrate immune-checkpoint sensitivity and evaluation of tumor–immune interactions via RNA-seq to better understand changes in TME and develop therapeutic combinations [60].

The group of Jenkins et al. [63] cultured tumor spheroid in collagen hydrogels suspended in 3D chambers where the response to PD-1 blockade was profiled. Fluorescence live/dead staining using dyes was performed to demonstrate the CD8 T-cell mediated killing on tumor spheroids treated with isotype control IgG antibody or anti-PD-1 antibody. Direct immunofluorescence labeling was also done using MDOTS and/or PDOTS. The CD8 staining enables identification of the effector CD8+ T cells present in PDOTS following anti-PD-(L)1 antibody treatment. In the case of Cañadas et al. [64], multiplexed immunofluorescence staining of cytokeratin, CD8 and CD4 was also performed in the platform. Their works showed that the 3D microfluidic culture maintains sensitivity to PD-1 blockade, whereas 2D culture using well plates are unable to detect the said response [60, 63–65]. Meanwhile, Sade-Feldman et al. [66] tried to determine which of the CD8+ T-cell state, CD8+CD39−TIM3− (DN, double negative) and CD8+CD39+TIM3+ (DP, double positive), can be used as a predictor for the success or failure of checkpoint immunotherapy. They isolated MDOTS from CT26GFP+ tumors of BALB/cJ mice, suspended it in collagen, and injected it into the gel region of the 3D microfluidic culture device. CD8+ T-cell DN and/or DP state were incubated as sole population or mixed one in the device together with MDOTS and anti-PD-1 (or anti-IgG control antibodies) for 5 days. The results showed that DN cells supports antitumor activity of the ex vivo checkpoint blockade [63, 64, 66]. The group of Rashidian et al. [67], on the other hand, made use of the system to aid in modeling TME and determine the distinction between responders and non-responders to anti-PD-1 treatment (anti-PD-1 IgG or an isotype control IgG) via CD8 immuno-positron emission tomography (PET). MC38 cancer cells, fibroblasts, and immune cells were used in the culture with the number of dead MC38 accounted for after 6 days. The effectiveness of the treatment, however, was assessed using PET with the distribution of the CD8+ T cells. Together with single-cell RNA-seq from intratumoral CD45+ and CD11b+ cells in responders and non-

responders, it was shown that anti-PD-1 treatment affects interaction of CD8+ T cells with tumor as well as impacts the intratumoral myeloid compartment [67]. Further work and development on these microfluidic platforms using MDOTS/PDOTS in aid or in combination with other techniques could lead to the selection and optimization of the best combinations of treatments, whether of small molecules, chemotherapy, or other immune agents in combination with ICB.

Other microfluidic platforms that were developed to monitor checkpoint blockade treatment response were designed as antibody delivery system or for CTC isolation. One example is the microneedle-array in a microfluidic chamber device assembly (SOFUSA^{TM}, Fig. 3-V), which was used by Kwon et al. [68], to deliver anti-CTLA-4 antibody to an orthotopic mammary carcinoma murine model and to human volunteers. The use of the said device allows the researchers to compare the antitumor response of the checkpoint blockade treatment strategy to that of the conventional IV Infusion approach. Here, near-infrared fluorescence imaging was utilized to image the lymphatic delivery of indocyanine green. The nanotopographic infusion capability of the device results to a better inhibition of tumor growth, arrest of metastases, increase in tumor-infiltrating lymphocytes, and complete response, as compared to the conventional systemic administration [68, 69]. Meanwhile the CTC-iChip (Fig. 3-VI) was used by Hong et al. [70] to isolate melanoma CTCs from whole blood of patients undergoing immune checkpoint inhibition therapy. Using microfluidics to enrich CTC and RNA-based droplet PCR for early tumor response quantification, melanoma cells in blood can be identified and accounted for through its 19-gene digital RNA signature (CTC score). This enables a highly sensitive, noninvasive, and blood-based monitoring of tumor burden. The results showed a decrease in CTC score and an improvement in progression-free survival and overall survival of the patient cohort under immune checkpoint inhibition therapy [70]. A summary of the advantages and disadvantages of the discussed microfluidic technologies in ICB treatment and related clinical application is presented in Table 1.

The use of microfluidic systems for ICB has several advantages, some of which have already been mentioned in the preceding discussions. Aside from being a noninvasive technique, integration of several technologies, such as surface enhanced Raman scattering for specific and multiplex detection of immune checkpoint proteins has already been demonstrated. Compared to conventional methods, the use of microfluidic systems allows direct perfusion of vascularized structures, increased control of microenvironment, and real-time and improved imaging capabilities [59]. Thus, it can provide greater insight and precision than existing preclinical tools. It is of small size, requires fewer samples and drugs, and can be mass-produced, thereby being cost-effective and reproducible [17]. The incorporation of microfluidic systems in clinical pipeline can reduce time and cost for personalized treatments in immunotherapy. This is exemplified by successful start-ups to commercialize microfluidic devices for single-cell analysis such as the C1 system (Fluidigm) and chromium system (10× Genomics) [71]. Advancement in microfluidic platforms are continuously being sought to improve the immunotherapy outcomes, particularly in immune checkpoint blockade. These advancements have transformed the way researchers and clinicians conduct research with great improvements in technique efficiency, data collection, analysis, and reproducibility. This has also led to collaborations with other fields of science and engineering as interest in automation and robotics, cloud computing, machine learning, and artificial intelligence builds.

Future of microfluidics in immune checkpoint blockade

The potential of immune checkpoint blockade therapy to induce durable responses across multiple types of cancer is undeniable as shown by its progression for the past years. However, it has been demonstrated that only ≤20–30% of tumor patients (melanoma, NSCLC, and renal cell carcinoma) have benefited from PD-1 or CTLA-4 antibody treatment. With a number of cancer patient populations still uncured, in addition to the high cost of treatment and toxicities that the unresponsive patient has to endure, better therapeutic solutions are needed [7, 11]. To predict the response of the patients and determine who can be saved by the immunotherapy, specific prognostic indicators requires urgent identification. This shifts the focus on identifying robust biomarkers in biopsies via immunological analysis. To help realize these, many researchers have turned to the use of various technology platforms.

It has been shown in high-dimensional single-cell analysis that immuno-profiling can predict response rates of patients treated with PD-1 inhibitors [11, 72]. Microfluidics platforms has the potential to aid clinicians and researchers in this search. A number of studies have already shown the capability of these tools to do single-cell profiling and analysis which can elucidate the role of the biomarkers in triggering or arresting antitumor immune responses [11, 23, 73, 74]. Integration of microfluidic techniques such as antibody barcoding and multiplexing, RNA-based droplet digital PCR quantification, indexing droplets, microchamber/well multiple displacement amplification, etc. can be used to inspect biopsies through genomic and/or transcriptomic analysis, provide information on the mechanisms that restrict the efficacy of the treatment, and give insight

Table 1 Advantages and disadvantages of microfluidic devices for therapy assessment and disease monitoring.

Microfluidic platform	Discussed application	Advantages	Disadvantages	References
Emulsion droplet microfluidic device	Screen and discover new anti-PD-1 antibodies in wild-type mice	Ultra-high throughput Can identify and isolate hits in an automated manner using fluorescence-activated sorting More comprehensive and less turnaround time as compared to conventional well-plate screening Can achieve native Ig pairing, capable of rare antibodies discovery, lower false negative	Require high number of starting material (>10,000 cells) Media exchange is difficult or not possible Difficult to perform assays requiring proliferation due to limited nutrients present in each droplet Does not allow long-term culture or complicated long-term culture process	[57, 82]
Valve-based microfluidic chip for single-cell compartmentalization	Quantification of single-cell granzyme B protein expression	Require small volume of samples and reagents High level of control, capable of media exchange and complex washing steps Capable of single-cell analysis, long-term and multiplexed monitoring	Limited throughput Bulky and complex external control equipments (i.e., motor pump)	[56, 82]
Nanotopographical microneedle-array in a microfluidic chamber device assembly (SOFUSA)	Deliver anti-CTLA-4 antibody to an orthotopic mammary carcinoma murine model and to human volunteers as well	Direct and controlled intracellular delivery of agents/drugs Can deliver very small amounts of drugs Sustained drug release, ease of use, and painlessness	Cost prohibitive and difficult to scaleup Limited dosing	[68, 69, 83]
Multiplexed immune checkpoint biosensor (MICB)	Parallel detection of PD-1, PD-L1, and LAG-3 in human serum	Parallel detection and simultaneous analysis Requires small sample volume Multiplexing capability, sensitivity, and relative assay simplicity Does not require expensive instrumentation	Low throughput Limited sample Volume Complex fabrication	[58, 84]
3D microfluidic cell culture system	To evaluate the immune-checkpoint sensitivity of murine or patient-derived organotypic tumor spheroids (MDOTS/PDOTS)	Requires small sample and reagent volume, Micro-scale dimensions compatible with in vivo system environments Real-time imaging Greater level of precision in TME interrogation and complex biological phenomena evaluation	Variability in number of spheroids within device Low reproducibility and data variability (PDOTS); high cost (MDOTS/PDOTS) Difficult to dispense cells in hydrogels and change growth media Difficult to collect cells after 3D formation; needs cell sorting to collect protein lysate and RNA from each cell population Needs experienced operators	[60, 85]
CTC-iChip (microfluidic positive immunocapture)	CTC isolation from the blood of ICB therapy patient	Whole-blood processing, high throughput Fast, high CTC count, low CTC burdens Various CTC-specific antigens can be used Cells are unaltered and viable after capture	Low purity of captured CTC Requires advanced knowledge on microfabrication	[86–88]
CellSearch (immunoaffinity based)	CTC detection, quantification, and enrichment in patient's blood	FDA-approved technique Multi-day specimen preservation, suitable for central laboratory testing Can predict the prognosis, for metastatic breast, prostate, and colorectal cancers patients	Cannot process whole blood Limited imaging and image analysis (intensity-based image analysis is not feasible) Low cell recovery, diminished viability EpCAM-positivity dependent	[12, 34, 87, 89]
IsoFlux system (microfluidic, immunomagnetic capture)	CTC enrichment and isolation	Higher sensitivity in CTC identification (as compared to CellSearch) Automated, continuous flow, low volume transfer requirement Intact viable CTCs, capable of cell retrieval, high recovery rate	Requires sample preprocessing to reduce volume Long, time-consuming process	[34, 87, 90]

Table 1 (continued)

Microfluidic platform	Discussed application	Advantages	Disadvantages	References
NanoVelcro (nanomaterial-embedded diagnostic platform)	CTC identification, capture, and release	Enhanced affinity and contact frequency with CTCs Scalable, reproducible, cost effective High sample-processing capacity, high-efficiency capture and release Can purify CTC without damaging RNA transcripts	Requires sample preprocessing to reduce volume Long, time-consuming process Less informative about EpCAM-low cells	[12, 90–92]
ExoSearch chip (microfluidic continuous flow platform)	Exosome capture and multi-marker probing	High flow rate and rapid isolation of exosomes High specificity in isolation of circulating exosomes Sensitive multiplexed exosomal marker detection Simple, cost-effective, robust	Requires sample pretreatment Can only separate exosomes that contain the targeted protein	[12, 38, 93, 94]
ExoChip (functionalized (CD63) multi-chamber/channel device)	On-chip exosome isolation, detection, quantification, and recovery	Increased exosomes' retention time, Can perform minimally invasive exosome-based quick diagnostic test Capable of simultaneous processing of multiple samples Design suitable for standard readout instruments Low fabrication complexity	Requires sample pretreatment Difficult to release captured exosomes Intact and label-free for downstream analysis	[39, 95]
nPLEX-nano-plasmonic exosome sensor (periodic nanohole arrays)	Simultaneous isolation and surface plasmon resonance (SPR)-based multiplexed detection	Scalable, high sensitivity, label-free profiling of exosomal proteins Fast, high-throughput, parallel exosome analysis	Limited to detecting transmembrane or lipid-bound proteins Expensive and sophisticated fabrication of nanostructures	[41, 95, 96]

into the wide spectrum of patients and their response [11, 75, 76]. From here on, target cell extraction can be incorporated to retrieve cells of interest, which can be further expanded or cloned for additional investigation, to have a broader understanding of the T-cell cytotoxic property and tumor immune evasion mechanisms, leading to personalized immunotherapy and overcoming of treatment resistance [24, 56].

These platforms could provide high volume and parallel throughput for rapid characterization at the time of diagnosis that can possibly tell how the patient will respond to a potential mAb treatment or fine-tune the desired immune response. Thus, future developments of this technology can provide solution to the challenge of effective and efficient assessment of the immunotherapy or therapy combinations in early-phase clinical studies. For instance, this can bring clarity and understanding of the differential effects of agents, such as PD-1/PD-L1 inhibitors, on the inhibition removal of exhausted effector T cells in the TME, the lifting of restraints from cancer-specific T-cell proliferation in draining lymph nodes, and on the effects on T-cell priming and activation [77].

The 2D and 3D microfluidic platforms for in vitro tumor models have the ability to replicate physical and spatial characteristics of TME [78]. The use of tumor organoids to these platforms allows the recapitulation of the immune checkpoint inhibitor response (i.e., anti-PD-1 antibody). It has the potential to optimize the efficacy of immunotherapies, test therapeutic combinations, as well as assess novel approaches. In particular, MDOTS/PDOTS in 3D microfluidic cultures can mimic the therapeutic sensitivity and resistance in vivo to PD-1 blockade via the assessment of tumor-infiltrating lymphocyte against tumor cells [79].

Although conventional approach using Matrigel culture for patient-derived organoids is promising for evaluating functional responses, limitations arises with the need for reproducing validation cohorts, effects of tumor heterogeneity, rapid real-time analysis, and correlation against patient response and overall survival [79]. Meanwhile, microfluidic culture for PDOTS-retaining tumor cells and autologous immune components provides a short term (i.e., 1 week) evaluation of cytokine profiling and T-cell cytotoxicity against tumor cell [79]. Organoid vascularization can be achieved in compartmentalized microfluidic chip, which is difficult to do in ordinary culture dish. Thus, represents the potential to predict patient's response to ICB therapy and possibly clinical translation from identification of optimally responsive cohorts. Furthermore, future developments and optimizations of these platforms could increase modeling sensitivity to reveal underlying resistance pathways that could lead to real-time resolution of patient sensitivity to ICB or combination of treatments.

This means that these new tools must be able to improve the efficacy of existing immunotherapy or identify more potent approach, at the same time reduce toxicity and adverse effects. For instance, microfluidic technology can provide novel and accelerated intracellular delivery strategy for macromolecules using nanoparticles, hydrogels, etc., to accommodate different therapeutic agents, based on patient-specific target, which can improve the efficacy of immunotherapy [80]. In addition, these devices must be able to provide ultrasensitive, accurate, and comprehensive assays for patient monitoring, before treatment, after treatment, and during remission. It must be able to conduct multiple tests in a singular chip. Furthermore, these tools must allow immunotherapy to reach a greater number of patients by being low cost and easy to use [12, 17, 53].

However, to realize these possibilities, several limitations and technical challenges of the current technology must be addressed. One big challenge is how to make microfluidic technology a mainstream in biological and clinical research, particularly its application to ICB therapy. Microfluidic devices are envisioned to facilitate the development of personalized medicine. However, many research works on microfluidic platforms are mainly proof-of concept studies and mostly published in engineering journals as opposed to clinical journals. Increased collaboration and interdisciplinary approach between engineers, scientists, clinicians, as well as organizations from the CIT community are needed to produce devices that are clinically relevant. This would require validation of the findings from models by comparing it to in vivo results and conversion to automated data analysis and readouts for routine clinical use.

To achieve this, microfluidic platforms have to diverge from heavy dependence on high-resolution imaging and time-consuming image analysis. Clinicians could use this technology to build patient-specific platforms using patient tumor and immune cells, and asses the efficacy of the immunotherapy prior to treatment. A high-throughput and automated microfluidic device integrated with various components (i.e., CTC isolation and diagnostic test in one device) could be readily available for physicians to use in the future.

Another challenge for this technology is mass production. Majority of the microfluidic devices are made up of polydimethylsiloxane (PDMS), which is limited in terms of manufacturability and durability. There are, however, alternative materials that can be used. Current designs and fabrication techniques are time consuming. Platform operation is still bench-top that needs external equipments (i.e., pumps, pressure gauges, microscope), several tubing and wirings, and a lot of human supervision. Although the integration of artificial intelligence such as machine learning and computational modeling could make analysis faster, most of the microfluidic platforms are still far from clinical application.

Continued advancement in the technology should be made to create integrated chips that includes more portable external devices and smart procedures that requires less human operation. External systems should be limited whenever possible [81]. For example, laboratory hardware like syringe pumps can be removed by incorporating gravity driven flow or capillary action in the design. Likewise, end-user experience must be improved for ease of use in the clinical setting. The realization of organs-on-chip or body-on-chip that mimic multiple organs in different compartments of a single device would not only allow us to predict responses to immunotherapies but could enable discovery of drugs or treatments that can cure majority if not all types of cancer, in the future.

In addition, commercialization of these diagnostic devices is also a challenge, as a balance must be sought between the scope and depth of its impact in immunotherapy, particularly in ICB, and the revenue that it can generate. Microfluidic devices can be pitched as a consumable in which multiple laboratories can integrate in several single-cell analysis applications. For example, droplet microfluidic chips sold by Dolomite and Fluigent, and used in laboratories for research applications still have to be integrated with downstream analytical methods. On the other hand, some companies such as μFluidix and microfluidic Chip-Shop provide facilities for customized microfluidic device fabrication, whereas others such as Sphere Fluidics provide complete integrated analytical solutions. Meanwhile, 10× Genomics allows profiling of immune cells at single-cell level with high automation and parallelization [13]. These are few examples of the many companies that have commercialized microfluidic technology for research purposes. Future endeavors would allow commercialization of these devices as a ready-to-use product that will suit the needs of immunotherapy research and clinical applications.

Conclusion

Finding a cure for cancer has gone a long way. From conventional chemotherapy and radiotherapy to the use of immune checkpoint inhibitors in immunotherapy. However unprecedented, the search for the cure is still a long way, as the efficacy and response rates of the treatment strategies are still low. Combination therapies, new biomarkers, and inhibitory receptors could potentially provide better results and greater coverage. The use of microfluidic devices for studies in immune checkpoint blockade provides new possibilities for an effective CIT. Microfluidic platforms can potentially evolve from a bulky laboratory setup to a more portable instrument that can be implemented on a larger scale outside of lab benches and into clinical bedsides. The continued innovation in this field could allow a shift from proof-of-concept stage to clinical application.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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