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Integrating Immunology and Microfluidics for Single Immune Cell Analysis

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The field of immunoengineering aims to develop novel therapies and modern vaccines to manipulate and modulate the immune system and applies innovative technologies toward improved understanding of the immune system in health and disease. Microfluidics has proven to be an excellent technology for analytics in biology and chemistry. From simple microsystem chips to complex microfluidic designs, these platforms have witnessed an immense growth over the last decades with frequent emergence of new designs. Microfluidics provides a highly robust and precise tool which led to its widespread application in single-cell analysis of immune cells. Single-cell analysis allows scientists to account for the heterogeneous behavior of immune cells which often gets overshadowed when conventional bulk study methods are used. Application of single-cell analysis using microfluidics has facilitated the identification of several novel functional immune cell subsets, quantification of signaling molecules, and understanding of cellular communication and signaling pathways. Single-cell analysis research in combination with microfluidics has paved the way for the development of novel therapies, point-of-care diagnostics, and even more complex microfluidic platforms that aid in creating *in vitro* cellular microenvironments for applications in drug and toxicity screening. In this review, we provide a comprehensive overview on the integration of microsystems and microfluidics with immunology and focus on different designs developed to decode single immune cell behavior and cellular communication. We have categorized the microfluidic designs in three specific categories: microfluidic chips with cell traps, valve-based microfluidics, and droplet microfluidics that have facilitated the ongoing research in the field of immunology at single-cell level.

Keywords: immunoengineering, microfluidics, single-cell analysis, cellular heterogeneity, cellular communication

INTRODUCTION: IMMUNOENGINEERING

The human immune system recognizes myriads of environmental triggers and is highly flexible in generating a variety of signaling responses over time (1, 2). Several types of cells collaborate with antibodies and cytokines to generate an appropriate immune response (3). The spatial organization and migration of cells within tissues as well as the dynamic nature of cellular communication enhances the complexity of our immune system and determines the type of response (4–7). The nature and magnitude of an immune response is dependent on dynamic

molecular and cellular interactions where well-orchestrated cellular communication is the key factor to maintain it (8). The question arises whether all immune cells fight all pathogens and tumors similarly in order to leverage this broad flexibility and diversity. Even though there are multiple subsets of immune cells, with each subset responding to specific stimuli, responses are often initiated by individual cells within each subset and communicated in order to establish a more complex population level response (9). Stochastic expression of genes (influenced by a cellular microenvironment) or pre-defined molecular drivers (as in case of B-cell and T-cell receptors) are driving factors behind heterogeneity in the human immune system (9). Numerous studies over the last decades established that heterogeneity is a trademark of the human immune system (10, 11). The identification of heterogeneity requires systems beyond conventional biological methods like ELISA, western blot, and others that do not allow the required spatial and temporal manipulation of biological cells (12–14). Technologies such as microsystems and microfluidics, have allowed scientists to study the individual behavior of immune cells, identify signaling pathways, map distinct immune cell subsets, quantify secreted molecules and characterize the immune response under varied conditions (15–17). Research on immune cells with technology integration has contributed toward innovative immunotherapy-based treatment modalities with lower treatment-related toxicity and side effects (18–20). They provide better alternatives to more conventional treatment modalities e.g., chemotherapy, radiotherapy or targeted therapy. At the same time, microfluidic based models have assisted in the development of novel therapies, discovery of new drugs, and monitoring the clinical efficacy of new treatments (21–23). Microfluidic devices are also used for the isolation of circulating tumor cells from clinical samples for diagnosis, prognosis, and creation of patient-derived tumor models with the aim to develop and test personalized medicine (24–27). Furthermore, integration of assays, microarrays, and several sensor technologies has led to the development of several point-of-care devices for the early diagnosis of cancer by identification of cancer biomarkers (28, 29).
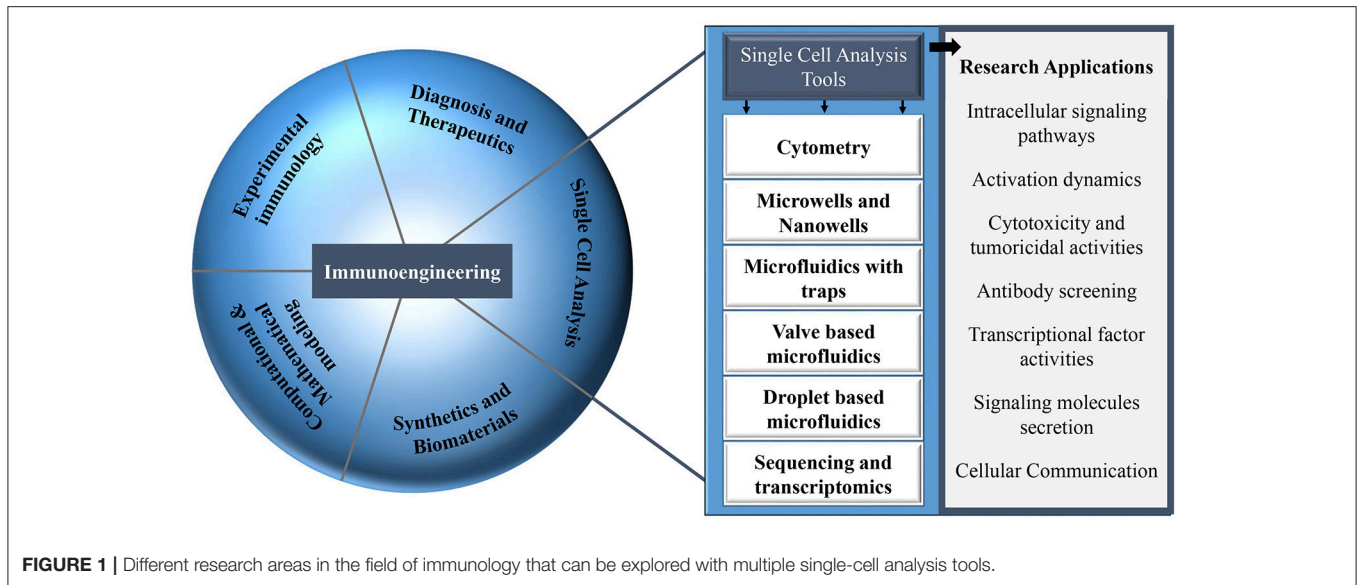
The majority of this type of research can be coined as immunoengineering. The term immunoengineering has been used since the seventies and covers several aspects in the field of immunology (30). Immunoengineering is an interdisciplinary and vast field of research comprising engineering methods and approaches that allow the modulation of the immune system and its responses: biomaterials, tissue engineering, protein engineering, synthetic biology, and drug delivery systems [**Figure 1**; (31–34)]. Immunoengineering includes the application of systems immunology to replicate complex immune microenvironment, *in vitro*, that aims to enhance our understanding of the human immune system for development of immunotherapy, the modulation of the human immune cells to boost their response against cancer (35–37). Recent

and most noteworthy examples of major discoveries within the field of immunoengineering, specially immunotherapy, are the development of chimeric antigen receptor T-cells and artificial antigen presenting cell systems (38–40). Moreover, immunoengineering also involves mathematical models that describe the functioning of the immune system, technologies to monitor and track the migration of immune cells and engineering tools to understand immune cell function at the systemic level in health and disease (41–43). The field of immunoengineering can, and has been described by various definitions, e.g., for the current issue in Frontiers "Application of systems immunology to engineering the tumor immunological microenvironment, aiming at predicting lymphocyte receptor's recognition patterns. Building sophisticated mimetic *in vitro* models, for instance by means of optical and magnetic tweezers to develop novel immuno-oncotherapeutics paving the way toward personalized and predictive medicine." The Center for Immunoengineering at Georgia Tech University defined this field as follows: "The field that applies engineering tools and principles to quantitatively study the immune system in health and disease, and to develop new therapies or improve existing therapies by precisely controlling and modulating a patient's immune response." The field of immunoengineering has been described in excellent reviews with a focus on engineering approaches to augment immunotherapy (44–48). In this review article, we highlight one aspect of immunoengineering and we particularly discuss various microfluidic and microsystems and focus on their advantages over conventional methods especially for decoding heterogeneous immune cell behavior and cellular interactions.

Single-Cell Technology

Immune cells, characterized by their heterogeneity, tend to differ in their behavior when in different societal contexts ranging from the single cell to the population level. Experiments performed at the population level average out the behavior of all the individual cells (49). Hence, bulk studies fail to provide a coherent understanding of the immune system by masking the phenotype, expressed genes, proteins or metabolites at single-cell level, and cellular communication between single immune cells (49, 50). The advent of single-cell technologies and the subsequent possibility to study the behavior of individual immune cells has uncovered various biological functions that were previously not detectable with bulk studies (51–53). For instance, Shalek et al. demonstrated the importance of paracrine communication for generation of immune response using single-cell analysis (54). Single-cell analysis enabled the investigation of maturation, activation, and signaling pathways of individual immune cells triggered by various environmental factors as well as intercellular communication between different immune cells (43, 55, 56). Additionally, this approach identified new immune cell subsets (57, 58). For instance, single cell transcriptomics, introduced a paradigm shift in the CD4+ T helper field and enabled the identification of multiple functionally distinct T helper cell subsets in addition to the two well-established subsets, Th1 and Th2 (59–62).

FIGURE 1 | Different research areas in the field of immunology that can be explored with multiple single-cell analysis tools.

Single-cell technology requires isolation of individual cells from a population for multiple data extraction from each isolated cell in order to gain information on the genotype, phenotype, lineage, protein secretion, proliferation, activation, maturation, cytolytic activity, and intercellular communication (63). Single-cell analysis tools are currently investigated by various research groups worldwide and hold great promise in providing a comprehensive understanding of our immune system. After the isolation of individual immune cells, multiple experimental operations for DNA sequencing, RNA and protein expression profiling can be implemented to map the lineage and identify subsets of immune cells (12, 64, 65).

Amongst immunologists, flow and mass cytometry are well-established, high-throughput, and high-content single-cell analysis tools (66–68). Flow cytometers measure fluorescently labeled cells and mass cytometers use transition element isotopes for mapping the functional heterogeneity and phenotypes of different immune cells by quantification of multiple cytokines, chemokines, and surface protein markers of the individual cells (69, 70). One of the benefits of cytometry over other conventional methods is its potential to provide high-throughput analysis of thousands of single-cells and measure multiple parameters in a given time frame (71, 72). Further, with recent advancements, mass cytometry can acquire samples using laser ablation to improve the resolution of this technology and is known as imaging mass cytomtery (73). Although cytometers are a powerful tool for single-cell analysis, spectral overlap and limited availability of antibodies labeled with isotopes for flow and mass cytometers, respectively are some of the drawbacks of this technology (13). Even though spectral overlap in conventional cytometry can be effectively mitigated by careful panel design, cytometers are still predominantly an end-point measurement tool that can only provide a snapshot in time and quantify static markers on cells to provide information on immune cell heterogeneity.

Microsystems for Single-Cell Analysis

The requirement for miniaturization of technological platforms has driven the development of several technologies such as microtiter plates (74). However, with microtiter plates, reaction volumes of immunological experiments have only been reduced from milliliters to microlitres. The problem of evaporation and capillary action in microtiter plate technology has hampered its further miniaturization (75). While microtiter plates cannot be further scaled down, the field of microsystems and microfluidics has played a key role in miniaturization to propel interdisciplinary research on single-cell analysis of immune cells.

Driven by the idea of scaling down, nanowells, in combination with microengraving, is a microsystems tool that was developed in the Love laboratory for single-cell analysis (76). Nanowells, made from polydimethylsiloxane (PDMS), contain features that have volumes in the order of nanolitres. When cells, from a bulk solution, are dispensed on the platform, individual cells settle down in each nanowell by gravity. Once the cells are isolated and activated, secreted molecules from the cells can be captured on functionalised glass slides (microengraving) or on the surface of nanowells and quantified by imaging cytometry or microscopy [**Figure 2**; (76, 77)]. Nanowell-based platforms with imaging cytometry and microarray analysis have been used for quantification of cytokines secreted by T-cells and observation of T-cell proliferation when activated with an array of ligands (CD80, major histocompatibility complex class II/peptide and intercellular adhesion molecule-1) or anti-CD3/CD28 (78). Nanowells can also be used for cell-pairing to study intercellular immune cell interactions and to monitor cytotoxic effector functions of immune cells (79). In 2017, An et al. presented their work on natural killer (NK) cells (80). In their study, they dynamically profiled the secretion of interferon gamma (IFN-γ) from single NK cells to map the phenotypic behavior of these cells based on their cytokine secretion pattern. Using this system,

they showed that CD56^{dim}CD16+ NK cells, when activated with phorbol 12-myristate 13-acetate and ionomycin, immediately secrete IFN-γ and that the secretion rate and amount of IFN-γ from these cells is dependent on the donor.

To study complex biological systems, it is essential that technology platforms replicate the cellular microenvironment accurately and also provide precise control over it. Microfluidic devices have been instrumental in providing automated platforms to perform all the essential functions for single-cell analysis of immune cells, on-chip. In the forthcoming sections, we discuss the contribution of microfluidics to the field of single-cell analysis, focused on immune cells, along with its advantages and disadvantages.

Microfluidics for Single-Cell Analysis

Over the last decade, microfluidics has made significant contributions to the field of single-cell analysis. This method allows cells to be monitored dynamically with a high degree of control over the cellular microenvironment (81, 82). These approaches have offered new information by creation of innovative conditions that are limited in conventional bulk methods. Microfluidic systems are being developed for applications in several areas such as protein purification and PCR on a drastically decreased scale (83, 84). Microfluidic chips are capable of accurately replicating *in vivo* biological environments and allow high-throughput analysis of cells (85). Microfluidics allows precise automation and control of analytical functions as well as manipulation of cells and their microenvironments with high resolution in both space and time (86, 87). With microfluidics, scientists can implement techniques and protocols for single-cell analysis through DNA sequencing, RNA expression, and protein quantification for understanding the mechanism of cell activation, proliferation, protein expression, motility and morphology, secretion, and cellular communication (88–91).

The ability to rapidly fabricate microfluidic devices in PDMS by soft lithography has greatly stimulated the development of several microfluidic designs (92). Besides being inexpensive, PDMS is biocompatible and permeable to gases, two properties that are a necessary for replication of artificial cellular microenvironments *in vitro* (93, 94). The flexibility of PDMS allows easy integration of membrane valves and pumps on more complex microfluidic designs to create an intricate network of microchannels wherein protocols can be realized in full automation with the help of programming software (95).

Microfluidic chip designs can be broadly classified in three categories: microfluidics with passive traps, valve-based microfluidics, and droplet microfluidics. With pros and cons of each design, in the field of immunology, microfluidics finds its applications in understanding immune cell behavior at single-cell level.
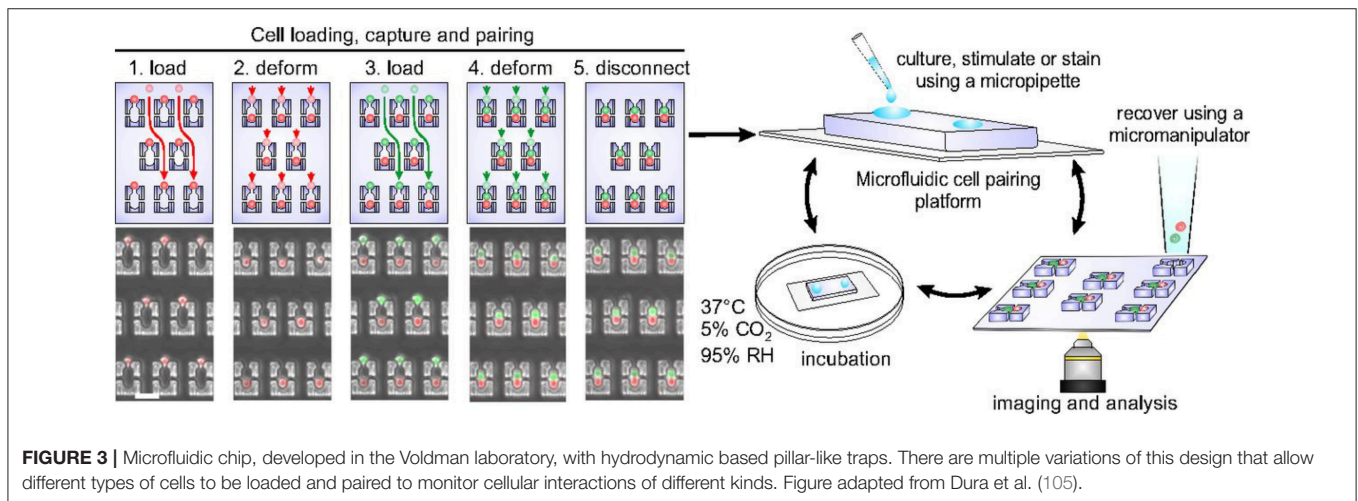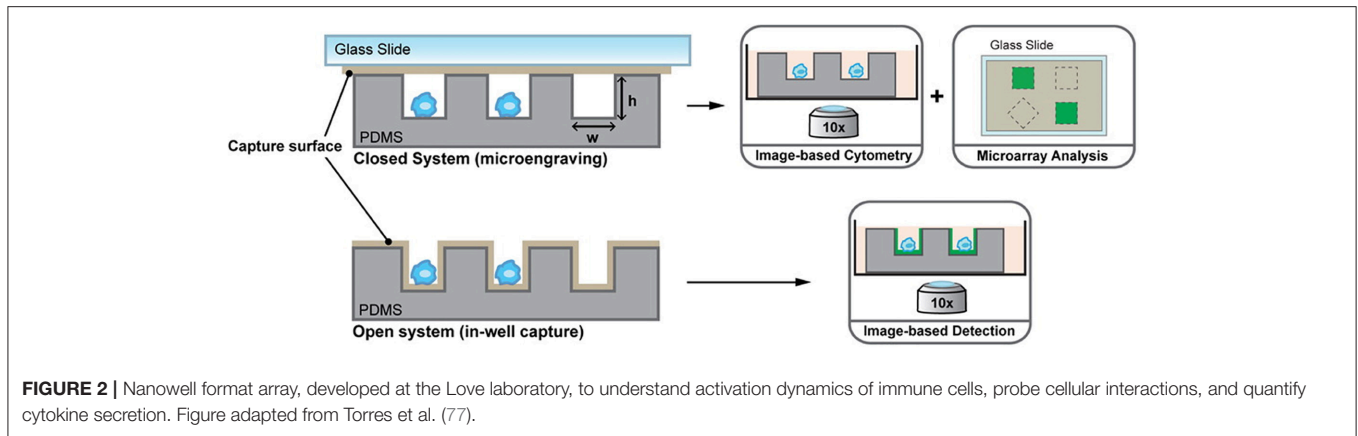
Microfluidic Chips With Cell Traps

Microfluidic chips with cell traps have been designed by multiple laboratories for single-cell studies (96, 97). In 2006, Di Carlo et al. designed a microfluidic chip with an array of hydrodynamic cell traps for analysis of enzyme kinetics in three different types

of cells (98). Later in 2009, Faley et al. presented their design of a microfluidic chip with multiple traps that was used to study signaling dynamics of isolated, individual, hematopoietic stem cells (99). Besides these studies, several groups have used hydrodynamic cell trap arrays for multiple applications in biology and chemistry (100–102). Of all these groups, the Voldman laboratory has extensively used a modified version of the hydrodynamic cell trap design for specifically studying immune cell interactions at single-cell level (103–106).

Cellular interactions play a vital role in establishing complex immune responses that originate from individual immune cells or immune cell subsets (107). Interactions between immune cells, if hampered, can cause several diseases (108). Hoehl et al. designed a single-layer microfluidic chip for parallel analysis of immune cell interactions at single-cell level (109). They used this design, integrated with weir like U-shaped traps, to pair two different cell types using a three-step loading protocol to obtain cell pairing efficiencies of more than fifty percent. The device was used for pairing murine T-cells with B cells to investigate the activation dynamics of T-cells. They demonstrated the presence of functional heterogeneity in the activation dynamics of OT-1 T-cells. Since OT-1 T-cell are reactive against ovalbumin, it is expected that all the T-cells will show similar activation profile when presented with the antigen. However, within the population of these cells, a variation in response was still observed even though all the cells bear the same identical T-cell receptor (TCR). Later, Dura et al. used this design to characterize the activation dynamics of CD8 T cells (OT-1 and TRP1) with different TCR affinities when paired with antigen presenting cells (104). This study showed that the variations in TCR affinity influences the secretion of cytokines by T-cells. The production of IFN-γ is strong for both low and high TCR affinity whereas the production of interleukin-2 (IL-2) reduces with reduction in the affinity. Dura et al. also used this chip to monitor cytotoxic effector functions of immune cells [**Figure 3**; (105)]. For this study, they modified the design of the cell traps to capture and pair NK92MI and K562 cells with high cell-pairing efficiency. The cytotoxic activity of NK cells was monitored by measuring the Ca^{2+} signaling for a day and further, the production of IFN-γ in NK cells, when activated with IL-2 and IL-18, was also quantified. Thirty-five percent of the cells showed cytotoxicity and 60% of the cell population produced IFN-γ over time, demonstrating cellular heterogeneity (105).

Besides applications in immune cell interactions, this design was also used for implementation of cell pairing and fusion protocols. In 2009, Skelley et al. paired fibroblasts, mouse embryonic stem cells, and myeloma cells, on-chip, to implement a more efficient electrical and chemical fusion protocol in comparison to the standard procedures (103). Further in 2014, Dura et al. modified the trap design to implement hydrodynamic and deformation based pairing and biologically, chemically, and electrically stimulated fusion of cells, on-chip (106). Kimmerling et al. in 2016 also used the principles of hydrodynamic trapping to design a microfluidic chip with an array of traps for the isolation of single murine leukemia cells, L1210, and primary CD8+ T-cells on chip (64). The isolated cells were cultured on-chip and,

FIGURE 2 | Nanowell format array, developed at the Love laboratory, to understand activation dynamics of immune cells, probe cellular interactions, and quantify cytokine secretion. Figure adapted from Torres et al. (77).

FIGURE 3 | Microfluidic chip, developed in the Voldman laboratory, with hydrodynamic based pillar-like traps. There are multiple variations of this design that allow different types of cells to be loaded and paired to monitor cellular interactions of different kinds. Figure adapted from Dura et al. (105).

after proliferation, these cells were released from the chip for investigating their transcriptomic profiles.

Microfluidic chips with cell traps have made significant contributions and have opened opportunities to investigate different immune cells to attain improved insights into cell-cell communication by allowing deterministic one to one cell pairing which is not easily attainable with other technologies.

Valve-Based Microfluidics: Microfluidic Large-Scale Integration Technology

Microfluidics large-scale integration (mLSI) is the integration of hundreds to thousands of pneumatic membrane valves, arrayed as fluidic multiplexers, on a microfluidic chip (95). Fluidic multiplexers, analogous to electronic multiplexers, allow complex manipulation of fluids with very small number of inputs. Valve-based microfluidic devices are made by aligning two separately cured PDMS layers with channels in such a way that the pneumatic membrane valves are formed when the channels in the two layers intersect each other orthogonally. These pneumatic membrane valves are "push down" when the control layer is on top of the flow layer and are "push up" when the alignment is reversed. The channels in the control layer are pressure driven and responsible for the actuation of pneumatic membrane

valves. The multiplexer on the chip helps in automation and parallelisation of experimental workflows and the pneumatic membrane valves on the chip help in fluid routing, metering, and control (110). mLSI integrated microfluidic devices are controlled using external units and can be programmed to operate for days, allowing them to monitor immune cell activity longitudinally (111).

mLSI devices are versatile and have been adapted for highly complex biological applications including cellular studies and genomic analysis (112–115). mLSI technology has been extensively used by the Quake lab for isolation of mRNA, synthesis of cDNA, and purification of DNA using fully-automated microfluidic chips (116, 117). Furthermore, they used a similar architecture for the ligation and transformation of genes with sample volumes of the order of a few nanolitres (118). In 2014, Ketterer et al. used a highly multiplexed microfluidic chip to develop a sensory system for quantification of metabolites from cellular samples (119). Blazek et al., from the same laboratory, implemented a proximity ligation assay on a fully automated microfluidic chip for analysis of phosphorylation kinetics in cells with high-throughput and parallel analysis (120, 121). The Maerkl lab also focuses on implementation of highly multiplexed and automated microfluidic designs for

characterization and quantification of transcriptional regulatory network and synthesis of genes and genome on microfluidic chips (122–124).

The generation of an immune response relies on environmental cues that are sensed by immune cells in their microenvironment (125). Signals received by cells within their microenvironment are not always continuous but often dynamic and can vary both in time, intensity, and concentration (126). More specifically, environmental cues can have variable amplitude, time of exposure, concentration gradient or can have pulsatile or sinusoidal variations (126). Among these variations, pulsatile modulation holds physiological relevance as several biological cues in our body such as hormones or cytokines are released with temporal variation that in turn affects the mode of action, downstream, of signaling molecules (127). In the human immune system pulsatile bursts of environmental signals dictate cellular heterogeneity and regulate the fate of transcription factors to influence the activation of genes and determine the phenotypic response of immune cells (126). Programmable mLSI based microfluidic devices make it possible to deliver pulsatile bursts of input stimuli to immune cells in a highly controlled fashion (128, 129). mLSI chips are capable of accurately mimicking the cellular microenvironment along with a reduction in extrinsic noise or cell-cell variability to generate synchronized immune cell responses at single-cell level.

In 2016, the Tay lab designed a fully-automated microfluidic device for studying the signaling dynamics of nuclear factor-κB (NF-κB) in macrophages [**Figure 4A**; (128)]. In their design they precisely replicated the dynamics of the immune cell microenvironment in a highly controlled manner and complete automation at single-cell level (128). NF-κB is an important transcription factor that is responsible for production of cytokines and survival of immune cells (130). The activation and deactivation of NF-κB shows oscillatory behavior and is popularly studied using microfluidics by providing immune cells with variable input stimuli (131–133). Junkin et al. used an mLSI based microfluidic system, which was integrated with a bead-based immunoassay, to investigate the transcription factor activity and quantify cytokine secretion in macrophages when stimulated with time variable inflammatory signals from the cellular microenvironment (128). The design comprised of 40, individually addressable, cell isolation chambers in which single immune cells were trapped using pillar like structures and each cell chamber was associated with its individual immunoassay unit. For the first time, this study showed the heterogeneous secretion profile of tumor necrosis factor (TNF) when single macrophages are simulated with dynamically variable input stimuli and that there is no correlation between the production of TNF and activation of NF-κB. Very recently an immunoassay was patterned on the microfluidic chip using a modified version of the mechanically induced trapping of molecular interaction (MITOMI) method for trapping antibodies to quantify TNF secretion [**Figure 4B**; (129, 134)]. Since the microfluidic device is based on the mLSI technology, it was possible to completely automate the experimental workflow including the patterning of the surface for an immunoassay. In this work, they were able to stimulate cells with dynamically variable signaling molecules in a
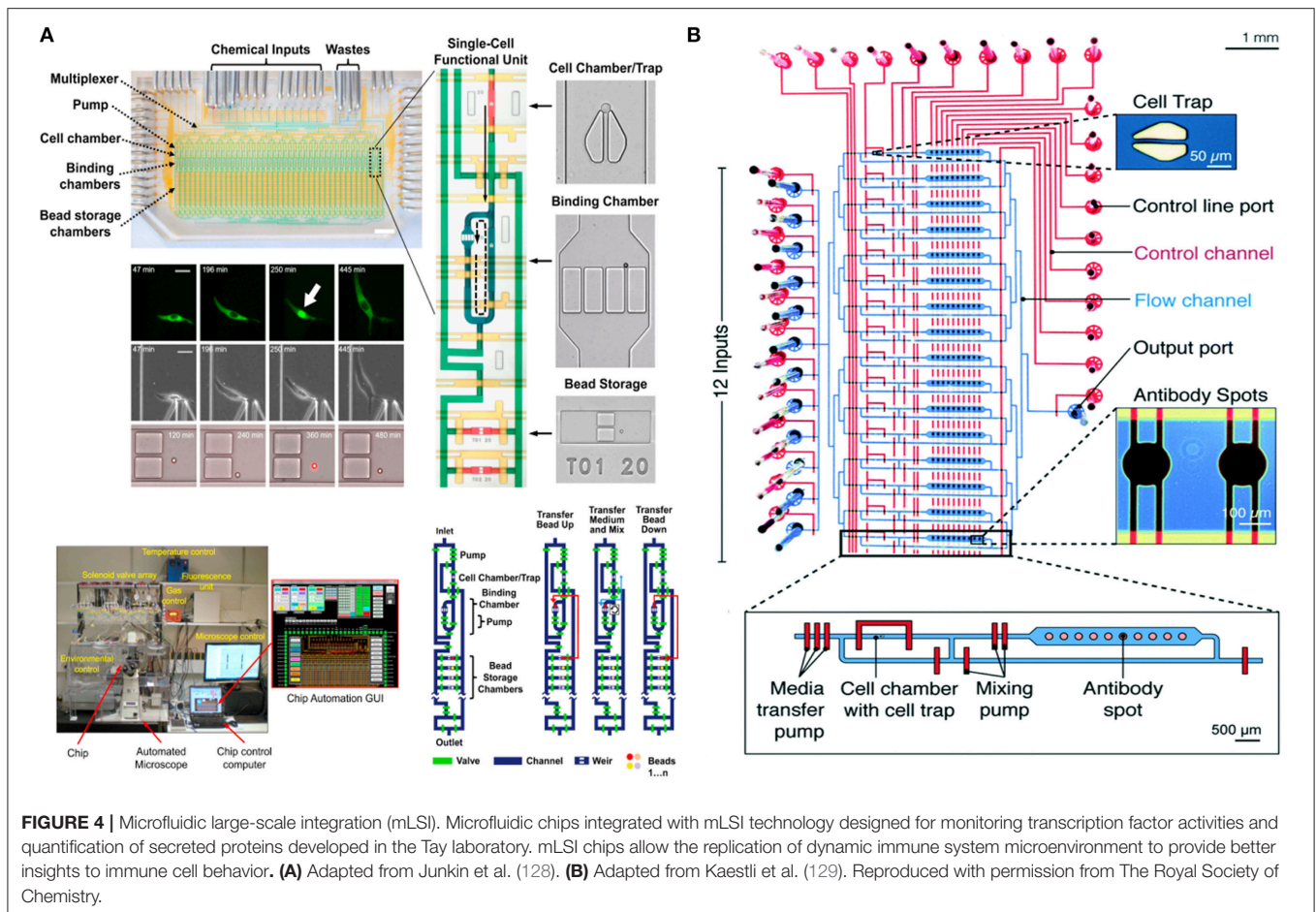
highly-precise and controlled manner as well as to monitor the activation of NF-κB in real-time using automated microscopy (129). Earlier, Frank et al. presented a microfluidic device that was used to co-culture macrophages and fibroblasts on-chip (135). This co-culture platform enabled the interaction of single immune cells with populations of cells. They used the automated device to provide dynamic inputs of lipopolysaccharide to single macrophages and monitor the signal transmission of TNF, upon activation of NF-κB, from single macrophages to a population of fibroblasts to replicate the initiation of the immune response. The experimental results of this work showed that an activated macrophage can spatiotemporally control the activation of NF-κB in fibroblasts to demonstrate that inflammation in tissues is regulated by the dynamics of gene expression (135).

mLSI technology allows automation of functional steps, on-chip, giving researchers the freedom to implement multiple experimental functions that are required for single-cell analysis. The aforementioned examples demonstrate that this technology has made valuable contributions to accurately replicate the dynamics of the cellular microenvironment with high precision and control. These designs can be further implemented to dynamically investigate the behavior of different immune cells at single-cell level.

Droplet-Based Microfluidics

The idea to perform biological analysis in water-in-oil droplets was first published in the 1950s by Nossal and Lederberg (136). Since then droplet microfluidics has continued to fuel a growing body of research leading to multiple applications in fields of biology and chemistry (137–139). Droplet microfluidics has been widely implemented for high-throughput screening of biological and chemical reactions, single-cell analysis, genomics, and transcriptomics (140–144). It also finds applications in molecular detection, imaging, drug delivery, antibody screening, toxicity screening, and diagnostics (145–151). On a microfluidic chip, using two immiscible liquids, droplets, in one liquid phase, are generated in another liquid phase by breaking off either at a T-junction or flow-focusing junction (152, 153). In such a setup, passive generation of droplets relies on drag forces and viscous dissipation (154). Variations in channel geometries help to pair, trap, merge, mix, release, and split droplets (155). Pneumatic membrane valves, electrical forces, optical manipulations and acoustic waves are other alternatives for active production of droplets on microfluidic chips (156–159).

Droplet-based microfluidic platforms provide scientists with the ability to investigate immune cell behavior in complete isolation by creating a noise-free and controllable cellular microenvironment (160). Specifically, it allows to map immune cell subsets, quantify the secretion of signaling molecules from single cells, and investigate cellular communication. In 2015, Sarkar et al. demonstrated an array-based droplet device that allowed monitoring of nanolitre-sized droplets for T-cell activation longitudinally right from the onset of activation (161). Their results suggested that the activation of single T-cells is faster when cells come in contact with dendritic cells in comparison to other activation methods. Furthermore, they developed a method to probe into the potentially heterogeneous cytolytic behavior

FIGURE 4 | Microfluidic large-scale integration (mLSI). Microfluidic chips integrated with mLSI technology designed for monitoring transcription factor activities and quantification of secreted proteins developed in the Tay laboratory. mLSI chips allow the replication of dynamic immune system microenvironment to provide better insights to immune cell behavior**. (A)** Adapted from Junkin et al. (128). **(B)** Adapted from Kaestli et al. (129). Reproduced with permission from The Royal Society of Chemistry.

of human NK cells (162). They demonstrated a 100% killing efficiency of NK cells, which is in contrast to earlier findings by various groups performed either in bulk or single cell (105, 163).

In order to quantify secreted molecules, cells are paired with functionalised beads or other sensing molecules to capture target analytes during incubation, prior to analysis (164). The droplet interface ensures that encapsulated cells are shielded from external factors that might influence their secretory behavior. Concurrently, this interface in combination with the small droplet volume, confines secreted molecules within the droplet resulting in increased sensitivity. Qiu et al. employed aptamer-based DNA sensors to quantify IFNγ secretion by encapsulating single T-cells in droplets followed by flow-cytometric and microscopic analysis [**Figure 5A**; (165)]. This study demonstrated the versatility of droplet microfluidics to be integrated with multiple detection methodologies. In another recent study, Eyer et al. used DropMap technology for phenotyping IgG secreting plasma cells at single-cell level [**Figure 5B**; (166)]. In this study, they paired antibody secreting cells with multiple paramagnetic functionalised nanoparticles that capture target antibodies in picolitre sized droplets. For the purpose of analysis, the generated droplets were immobilized in a glass observation chamber to measure fluorescence intensity of each droplet and to quantify secreted antibodies to map different

plasma cell phenotypes. With this technology it is possible to monitor and quantify antibody secretion by encapsulated cells in droplets real time.

Besides aqueous based droplets, hydrogel agarose can also be used to create droplets in oil phase, which allows washing steps and permits staining with antibodies within droplets by slow diffusion. This conceptual advantage of using hydrogel based droplets was exploited in the Huck laboratory, where agarose droplets were used for encapsulation of Jurkat T-cells to capture multiple cytokines on functionalised beads and used to demonstrate cellular heterogeneity and mapping cellular subsets [**Figure 6**; (167)]. Generally, for cytometry, droplets need to be broken to retrieve cells and beads. On the contrary, cells and beads encapsulated in hydrogels can be analyzed directly with flow cytometry, preventing loss of cells and saving significant amounts of time.

Recently, researchers have also implemented protocols for single-cell sequencing in droplet microfluidics (168). In 2015, Macosko et al. developed the Drop-seq technology where the transcriptomics of thousands of retinal cells were analyzed in droplets using barcoded microparticles (169). Later, the Abate lab also demonstrated the genomic sequencing of more than fifty thousand cells at single-cell level in agarose microgels (170). Single-cell sequencing allows researchers to
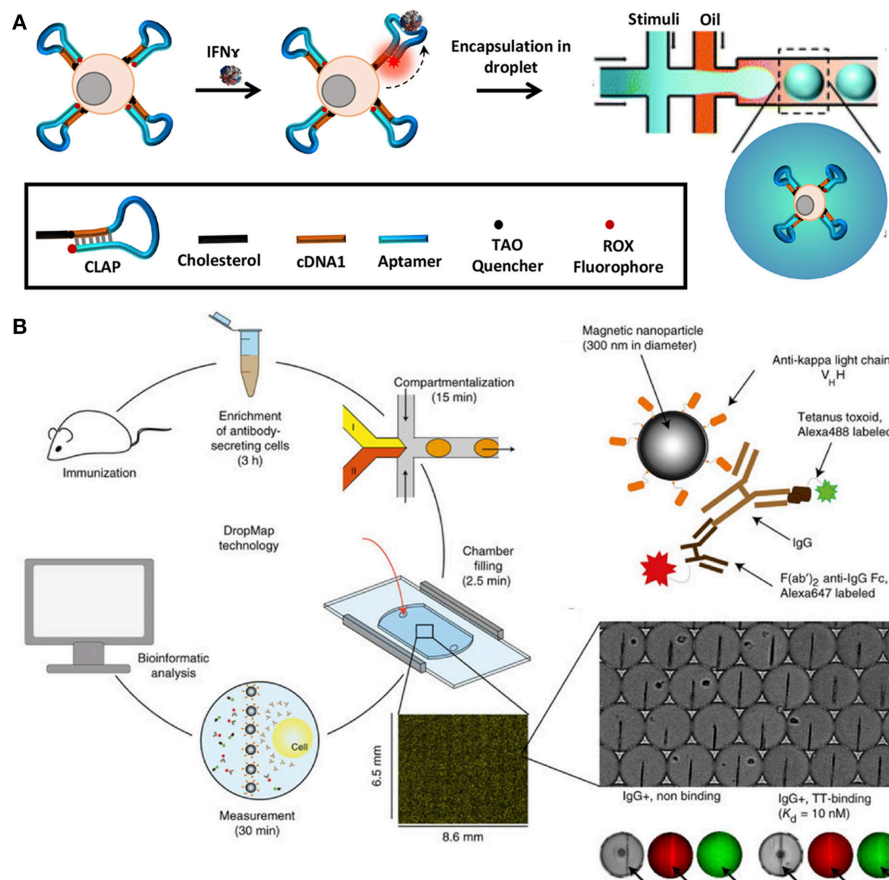
FIGURE 5 | Droplet microfluidics is a very versatile tool that allows single-cell analysis of immune cells in a noise-free environment. The cells are often paired with functionalized beads or sensors such as aptamer sensors for quantification of secreted molecules and proteins. Droplet microfluidics is combined with flow cytometry, mass cytometry, and automated microscopy for downstream analysis. **(A)** Adapted from Qiu et al. (165). **(B)** Adapted from Eyer et al. (166). Reproduced with permission from Springer Nature.
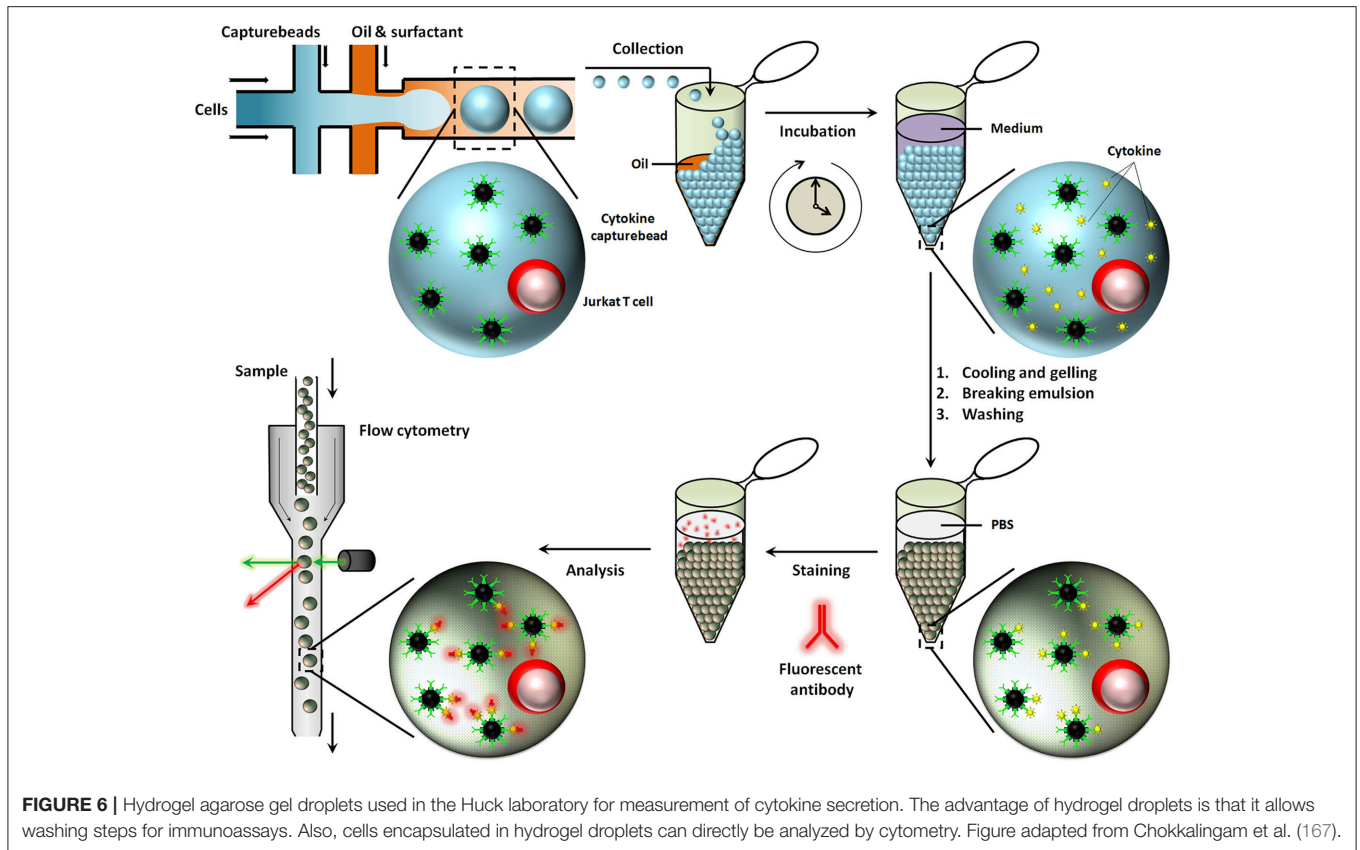
identify the differences in cellular behavior and understand the functionalities of individual cells, which assists in decoding immune cell heterogeneity (171). Genomic amplication for sequencing can be performed in droplet microfluidics with high accuracy and specificity in a massively parallel fashion (168). The work of Shahi et al. demonstrated the efficiency of droplet microfluidics to profile protein secretion by single immune cells using a high-throughput droplet-microfluidic barcoding technique, Abseq (172). This microfluidic device was integrated with functions to amplify DNA in nanolitre sized droplets to allow more than tens of thousands of cells to be analyzed in parallel.

Together, all these studies highlight the role of droplet microfluidics in single-cell analysis of immune cells. Droplet-based microfluidics is a highly versatile and flexible technology and is widely applicable in multiple realms of immunology. The ability to carry out high-throughput analysis of hundreds to thousands of individual immune cells and paired immune cells in a parallel manner makes droplet microfluidics a highly reliable and popular single-cell analysis tool.

Strengths and Weaknesses of Microfluidic Technologies

The motivation for miniaturization was driven by the requirement to acquire more information from single cells at higher resolution. Flow cytometric analysis allows for sampling cell populations in time but fails to provide dynamic information from single immune cells. Also, high costs of the equipment and infrastructure for mass cytometers often limits the usage of this technology. To compensate for the drawbacks of the cytometric analysis and gather more temporal information on the behavior of single immune cells, microsystems and microfluidics gained popularity. Innovation in microsystems and microfluidics facilitated the integration of numerous complex functions on-chip that were earlier not feasible or demanded a lot of manual labor. At the same time, the ability of these platforms to dynamically acquire information from immune cells and monitor immune cell activities real time made them popular among researchers.

Nanowells and microfluidic chips with hydrodynamic cell traps are the simplest examples of miniaturization that,

FIGURE 6 | Hydrogel agarose gel droplets used in the Huck laboratory for measurement of cytokine secretion. The advantage of hydrogel droplets is that it allows washing steps for immunoassays. Also, cells encapsulated in hydrogel droplets can directly be analyzed by cytometry. Figure adapted from Chokkalingam et al. (167).

because of ease of fabrication and operation, are frequently used for decoding immune cell behavior and intercellular communication. These high-throughput analysis platforms allow both real-time and end-point measurements and can facilitate one to one cell pairing for decoding communication between immune cells, e.g., for monitoring cytotoxic cellular function. However, microfluidic chips with cell traps are more efficient in achieving desired pairing efficiencies in comparison to nanowells. These platforms are limited by their ability to replicate the dynamically variable immune cell microenvironment in which immune cells work. Also, for more efficient single-cell level analysis of immune cells it is essential that cells are isolated and analyzed in a noise-free environment to negate the effects of paracrine communication from neighboring cells.

Valve- and droplet-based microfluidics have realized the aforementioned key requirements and have been able to circumvent the drawbacks of other single-cell tools. Both these platforms have the ability to compartmentalize cells in a closed environment to understand cellular behavior with high sensitivity. One of the key advantages of programmable valve-based microfluidics is that it allows the replication of dynamic immune cell microenvironments with high precision for delivering input stimuli in forms of pulsatile bursts. Although the process of fabrication and experimental setup for such devices is fairly complex and time-consuming, automation and reproducibility compensates for the drawbacks of these designs (132). Also, the throughput of mLSI designs is often

low to medium, but has the capacity to be increased by scaling.

For high-throughput analysis of immune cells, droplet-based microfluidics is preferred. Easy to design and implement for multiple applications, it allows the isolation of single immune cells in droplets for analysis in an isolated system. Small compartment size and very low droplet volumes makes this system highly sensitive by preventing the loss of stimuli and secreted molecules from the system. Further, this system also facilitates encapsulation of multiple cells in a closed environment to understand immune cell communication, e.g., for cytotoxic behavior. While droplet microfluidics is a well-established tool in the single-cell analysis community, it often finds limited applications with use of primary, rare immune cells because of the difficulties faced during seeding of cells. Traditional cell seeding methods often lead to a loss of the cells because of attachment or sedimentation, and when cells are already rare in population, it is difficult to obtain high encapsulation rates (173). Encapsulation of cells in droplets is random and relies heavily on Poisson statistics (174). To overcome the limitations of Poisson statistics alternative cell seeding methods as well as use of external physical forces are required to ensure desired cell distribution in droplets (175, 176). The designs discussed in this review, each with their own set of advantages and disadvantages, have been widely implemented for several single cell studies to enhance our understanding of various immune cell functions (**Table 1**).

TABLE 1 | Table summarizing different single-cell analysis tools discussed in this review in terms of their advantages, disadvantages, and applications.

	Research applications	Advantages	Limitation	Commercial vendors	References
Cytometry	Cytokine and surface markers Signaling and activation dynamics Cytotoxicity Immunophenotyping Sorting	High throughput Quick to run (on the order of several hours) Ability to sort cells based on expression of molecules	Spectral overlaps Expensive Not possible to replicate dynamic cellular microenvironment Static snapshot over time	BD Biosciences, MilliporeSigma, Miltenyi Biotec, Thermo Fisher Scientific	(177), (178), (179)
MICROSYSTEMS					
Nano wells	Antibody and drug screening Activation dynamics Cytotoxicity Inter and intra cellular communication Cytokines and signaling molecules release	High-throughput Possibility to recover cells from the wells Simple to use Multiplexing and real time monitoring Quantitative analysis of proteins Possibility to pair multiple cells in each well	Seeding cells under the effect of gravity lowers the efficiency of cell loading Manual operation Limited control over the fluidic and cellular microenvironment Random distribution of cells in wells	µFluidix, microfluidic ChipShop	(76), (77), (79), (80), (163), (55), (78)
MICROFLUIDICS					
Trap-Based	Inter and intracellular communication Cytotoxicity Signaling and activation dynamics Cytokines and signaling molecules release	Efficient cell pairing and fusion Ability to monitor cellular behavior, from the point of cellular interaction and contact Multiplexing and real time monitoring High throughput	Stimulation of cells in bulk and not on-chip (Juxtacrine and paracrine interactions before loading the cells cannot be ruled out) Controlled on-chip stimulation is not possible Cells are not isolated from each other (The trapped cells and secreted molecules are not completely confined and this can influence the behavior of the neighboring cells by paracrine communication)	µFluidix, microfluidic ChipShop	(104), (105), (106), (96), (109)
Valve-based	Signaling and activation dynamics Cytokines and signaling molecules release Monitor transcription factors activity Migration	Complete automation Replication of dynamic cellular microenvironment Sensitive (addressable cell chambers with very small volume) Low sample volume Both real time monitoring and end-point analysis	Laborious and time-consuming fabrication Not portable (additional equipment for operation) Low to medium throughput	Fluidigm C1	(128), (129), (135), (16), (180), (181), (182)
Droplet-based	Inter and intracellular communication Cytokines and signaling molecules release Activation dynamics Antibody screening Cytotoxicity	High throughput Noise-free cellular microenvironment due to compartmentalization Both real time monitoring and end point analysis Multiple as well as single cell pairing Sensitive (low reaction volume) Small sample and reagent volume	Not possible to replicate dynamic cellular microenvironment Random encapsulation (follows Poisson Distribution) Difficult to incorporate washing steps	µFluidix, microfluidic ChipShop, Dolomite, Fluigent, 10X Genomics (Chromium Controller) BIORAD (ddSEQ)	(144), (162), (164), (166), (167), (161)

The applications described here are what is presented in this review as well as all the other potential applications of the design for immune cell analysis at single-cell level.

CONCLUSION AND FUTURE OUTLOOK

Single-cell analysis tools have played a major role in enhancing our understanding of the human immune system. Several research groups have focused on technology development to constantly provide novel design alternatives for biological studies. Thereby, it enabled to address complex immunological questions that were earlier not possible with conventional bulk methods and resulted in identification of heterogeneous immune cell behavior, discovery of new immune cell subsets, and understanding how single immune cells drive population responses. Single-cell analysis facilitated the design and development of new diagnostic tools, personalized medicines, and immunotherapies for treatment of cancer, immunosuppressive diseases, and autoimmune disorders (183–186). For example, single-cell studies recognized specific signaling pathways within individual immune cells that were suppressed in a tumor microenvironment (187). Identification of such immunosuppressive signaling pathways, molecules, and individual immune cells improves the design of treatment modalities aimed at targeting cells and activation of the suppressed signaling pathways to fight cancer, infectious- and auto-immune diseases (184, 188). For development of vaccines, it is critical to understand how specific antigens induce effective immunization. Novel vaccines with higher clinical efficacy can be developed using results from antibody screening and quantification experiments at single-cell level (149, 166). Also, quantification of signaling molecules at single-cell level provides information on new pathways for development of sensitive diagnostic tools that can provide faster and accurate results in comparison to traditional laboratory methods (189).

Developments in the field of microsystems and microfluidics have been ongoing for more than a decade and continues to grow. As our understanding of the human immune systems deepens, more questions arise to decode the complexity of our system. To cater to these questions, technology continues to evolve. The designs discussed in this review were limited to applications in single-cell analysis of immune cells. However, there are several established and on-going design developments in single-cell research for multiple biological applications that can be easily modified and implemented for better understanding of the immune system. As an example, the microfluidic droplet system published by Shembekar et al. can be modified for use with primary B-cells for antibody screening (149). Furthermore, a droplet microfluidic system integrated with Protein Assay via Induced Gene Expression (PAIGE) can be used for quantification of secretory molecules at single-cell level (190). Microfluidic chips can be also be used to study immunosurveillance and migration of immune cells, *in vitro* (191, 192). Finally, there are several other designs that can be integrated with different systems or modified for immune system related research (193–195).

There are multiple small and medium scale companies that have commercialized microfluidics, as individual components or complete analytical system, to promote the integration of microfluidics in multiple laboratories for several single-cell analysis applications. Companies like Dolomite and Fluigent fabricate droplet microfluidic chips that can be bought and directly used in laboratories for research applications. These chips, however, still have to be integrated with downstream analytical methods. Other companies such as µFluidix and microfluidic ChipShop do provide professional facilities to fabricate different types of microfluidic devices as per the user requirements. Sphere Fluidics also provides completely integrated analytical solutions for single-cell research. Further, there are commercial systems integrated with microfluidics that provide complete analytical solutions to research problems. One such example is the C1, developed by FLUIDIGM, that is integrated with microfluidic circuits for transcriptomics at single-cell level to identify heterogeneity among immune cell population. This system provides a fully automated solution to implement experimental protocols with high precision and accuracy. The Chromium Controller by 10X Genomics allows profiling of immune cells and their repertoire at single-cell level with high automation and parallelisation. ddSEQ by BIORAD uses droplet microfluidics for isolation of single cells to provide sequencing solutions at single cell level. This commercial device has multiple applications including assessment of cellular heterogeneity, identification of cellular sub-populations, and functional analysis of immune cells. The aforementioned examples are just a few of the many companies that have commercialized microfluidic technology for research puposes.

Taken together, robust technology for decoding cell-cell or cell-pathogen interactions longitudinally and in great detail will revolutionize cell biology and the fields of immunology and cellular immunotherapy in particular. The impact of rapid expansion of single-cell analysis is evident in its great potential for numerous applications, including, but not limited to: cancer research, regenerative medicine, diagnostics, and synthetic biology. We believe that even though technology development might sometimes be an extended process, the ease and cost-effectiveness of microfluidics will boost the integration of this exciting technology in the portfolio of other single-cell assays used in cell biology and immunology related disciplines.

AUTHOR CONTRIBUTIONS

NSi, NSu, and JT conceived the manuscript and the writing of the manuscript and approved its final content.

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