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Review

Profiling signaling mediators for cell-cell interactions and communications with microfluidics-based single-cell analysis tools

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SUMMARY

Cell-cell interactions and communication represent the fundamental cornerstone of cells' collaborative efforts in executing diverse biological processes. A profound understanding of how cells interface through various mediators is pivotal across a spectrum of biological systems. Recent strides in microfluidic technologies have significantly bolstered the precision and prowess in capturing and manipulating cells with exceptional spatial and temporal resolution. These advanced methodologies converge with multi-signal mediator detection systems, furnishing potent, high-throughput platforms for dissecting cell-cell interactions at the single-cell level. This approach empowers researchers to delve into intricate cellular dynamics with unprecedented accuracy and efficiency. Here, we present a critical evaluation of the latest advancements in microfluidics-driven techniques for detecting signal mediators involved in cell-cell interactions and communication at the single-cell level. We underscore notable biological applications that have benefited from these technologies and identify pressing challenges that must be addressed in future endeavors leveraging microfluidic tools for single-cell interaction studies.

INTRODUCTION

Within the intricate tapestry of a multicellular organism, cells are not solitary entities but rather perform their designated functions within specific niches, engaging in intricate interactions with neighboring cells through both direct contact and a myriad of signaling mediators.[1–3] These cell-cell interactions are fundamental to a vast array of biological processes, encompassing cell division, proliferation, migration, differentiation, and apoptosis.[3–6] Conversely, dysfunctional interactions and communication among cells have been consistently implicated in the pathogenesis of numerous diseases, such as cancer metastasis, motor neuron diseases, virus-host interactions, and diabetes.[7,8] Nevertheless, unraveling the intricate mechanisms underlying these cell-cell interactions and communications remains a formidable challenge, primarily due to the intricate and sophisticated nature of intercellular interaction networks within microenvironments.

Besides direct contact, cell interaction and communication are orchestrated by the intricate binding of diverse ligands and receptors, encompassing cytokines, DNA, messenger RNA, proteases, and extracellular vesicles, among others (Figure 1).[1,9] These bindings elicit physiological responses vital to cellular functioning. Advancements in methods to study single-cell interactions and communications, particularly single-cell functional proteomics and RNA sequencing, have ushered in unparalleled opportunities to decipher cellular interactions at unprecedented resolution.[10,11] By quantifying ligand and receptor expression across cell types, high-throughput single-cell transcriptome sequencing technology predicts intercellular communication networks, leveraging ligand-receptor interactions as a cornerstone for unraveling cell-to-cell interactions. Illustratively, Mathys et al.[12] employed single-cell transcriptome sequencing technology to analyze neuron cells from Alzheimer's patients, revealing myelin formation's pivotal role in the disease's progression by contrasting ligand and receptor expression profiles across individual neurons.[12] However, single-cell transcriptome sequencing faces challenges, including low transcriptome-proteome correlation and temporal-spatial disparities across cells, which may skew the understanding of cellular interactions.[13] Direct detection of signaling mediators underlying cell-cell interactions is thus considered more reliable. Imaging techniques have evolved to visualize cell-cell signaling proteins like cytokines, albeit limited by spectral overlap that restricts the number of detectable markers.[14,15] Single-cell secreted protein detection technology, such as ELISpot[16] and flow cytometry with

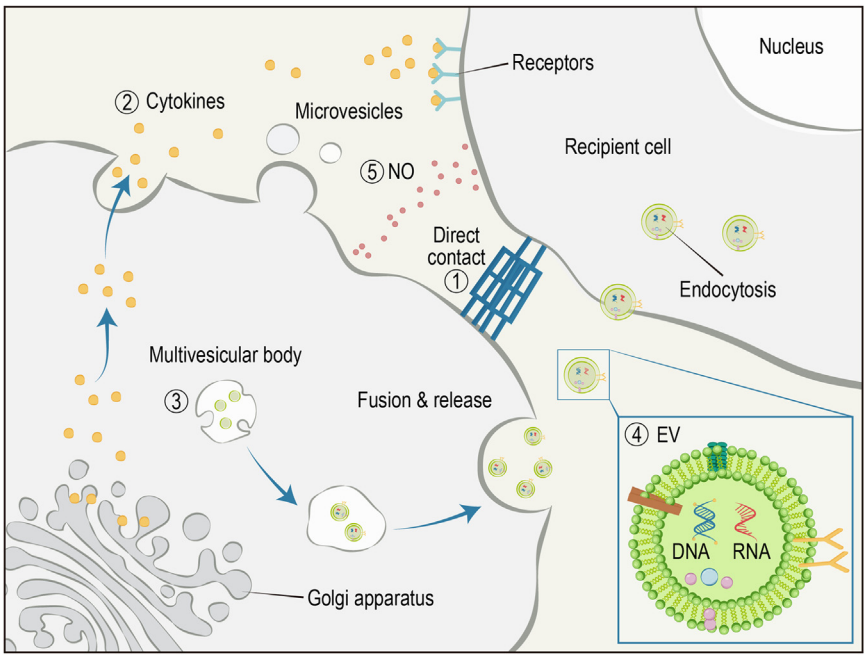

Figure 1. Cells engage in multifaceted interactions and communication via diverse pathways

including but not limited to: (1) direct contact, (2) signaling mediated by proteins and peptides such as cytokines, (3) exchange of extracellular vesicles, (4) transmission of nucleic acids like DNA or RNA, and (5) utilization of small molecules such as NO and CO as signaling molecules. These modalities underscore the intricate web of cellular communication and its critical role in maintaining homeostasis and orchestrating biological processes.

fluorescence or mass spectrometry,[17] overcomes this hurdle, enabling multi-dimensional analysis of secreted proteins to precisely quantify cell interaction's extent and intensity, facilitating functional protein-level insights.[18,19] Liao et al. utilized microporous arrays to study Alzheimer's neuron cells' interactions through single-cell secreted proteins, discovering that interacting cell counts did not impact Aβ and sAPPα secretion.[20] Similarly, Abdullah et al. developed a neuron-neuron interaction chip, demonstrating that interactions significantly upregulated the secretion of PDGF-aa, GDNF, and FGF-2 at the single-cell level.[21] Recently, exosomes have emerged as pivotal signaling entities, their multi-dimensional profiling offering profound insights into physiology and pathology, with implications for disease diagnosis and treatment.[22–27] Profiling these signaling mediators holds immense potential in addressing a myriad of crucial biological questions pertaining to underlying pathways.

The traditional methodologies employed for elucidating intercellular interactions and communication, notably co-cultured systems subsequently analyzed via mass spectrometry or microscopic imaging,[28] have indeed imparted substantial insights into the realms of cell biology and medical biology. Nevertheless, a preponderance of these techniques primarily relies on population-based cellular detection, limiting their scope. This trend obfuscates the diversity among cells and their intricate interaction patterns, emphasizing the necessity for the development of more efficient approaches to unravel intercellular interactions at the single-cell level. Microfluidic-based techniques for biological and medical investigations have recently gained practical feasibility and have been extensively applied in various fields, including vascular biology, tumor biology, as well as liver and nerve tissue engineering, among others.[29–32] A microfluidic system efficiently facilitates precise manipulation of single cells in a high-throughput fashion, rendering it an ideal platform for conducting single-cell experiments with unparalleled precision and efficiency.[33–35] Leveraging advanced cell manipulation technologies, including micropumps, microvalves, optical tweezers, and electrophoresis,[36–38] a range of cellular operations, from culturing and sorting to transfection and dissolution detection, can now be performed on the chip, streamlining the process and enhancing precision. Microfluidic chips possess the remarkable capability to emulate the intricate microenvironments found *in vivo*, enabling precise control over cells and their immediate surroundings.[39] Furthermore, they facilitate real-time monitoring of intercellular interactions within the chip, offering unparalleled insights into cellular dynamics.[40] This advanced technology also allows for the meticulous analysis of interactions between both homologous and heterologous cells, providing invaluable insights into their behavior and interplay. Moreover, the efficient acquisition and analysis of high-content data, crucial for scientific discoveries and conclusions, can be achieved through the utilization of automated counting and analysis tools.[41] This approach significantly streamlines the process, enhancing the accuracy and timeliness of research outcomes.[42] These tools have been widely embraced, for instance, in the isolation of circulating tumor cells,[43,44] exosomes,[45] single-cell sequencing endeavors,[46] single-cell secretion detection,[47] and numerous other applications.[48] Notably, microfluidic-based single-cell systems showcase unparalleled benefits such as minimal consumption, meticulous manipulation, high throughput, and seamless integration.[28,49] These systems demonstrate exceptional efficiency and sensitivity in analyzing cell-cell interactions, making them the tool of choice for biological and medical investigations.[50] Furthermore, a key advantage of microfluidic-based single-cell systems lies in their capacity to unravel the heterogeneity and unique interaction signatures of individual cells or specific subpopulations. This unparalleled ability to decipher nuances that remain elusive to other interaction study methodologies has the potential to yield previously unexplored insights, propelling the understanding of cellular behavior and interactions to new heights.

Microfluidic systems designed for studying single-cell interactions exhibit remarkable diversity (Figure 2), commonly incorporating microwells and microchannel arrays,[51–53] microdroplets,[54–56]

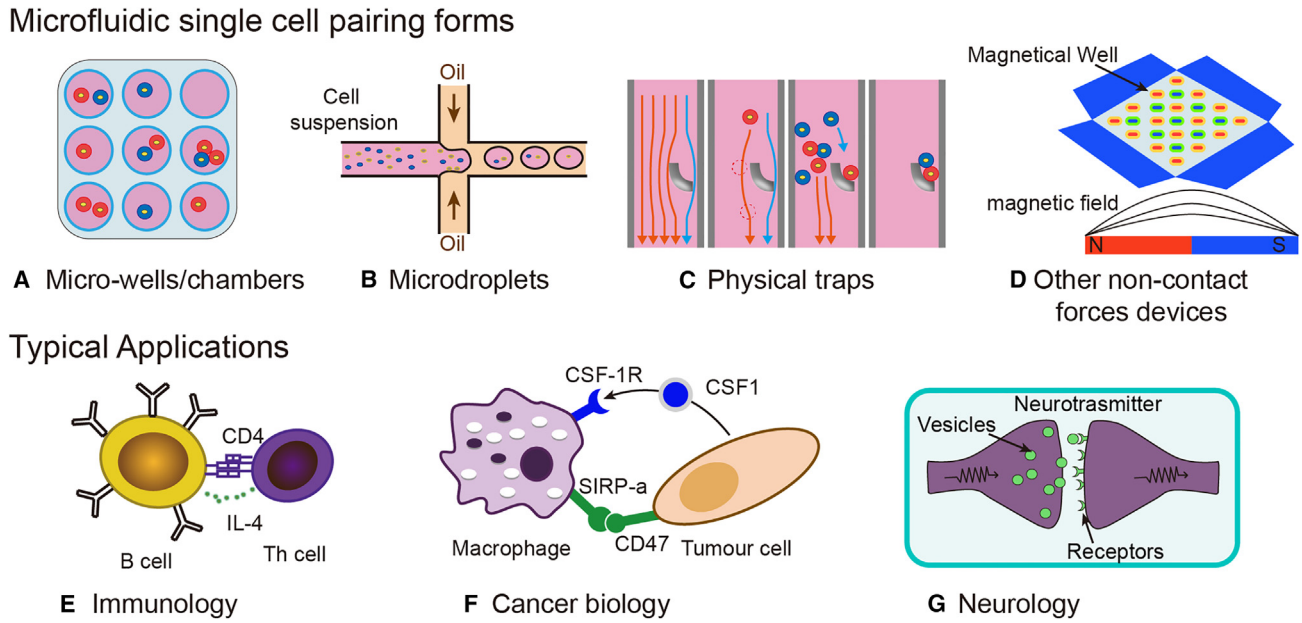

Figure 2. Schematic illustration of forms and typical applications of microfluidics-based single-cell analysis tools for cell-cell interactions

(A–F) Commonly used microfluidic single-cell pairing forms mainly include (A) micro-wells/chambers, (B) microdroplets, (C) physical trapping methods, and (D) devices based on non-contact forces such as magnetic field. These microfluidic devices have been utilized in various fields, including (E) immunology, (F) oncology, and (G) neurology.

fluidic trappings,[44,57–59] acoustic,[43] magnetic,[60] and optical devices.[61] Each of these systems possesses distinct advantages and limitations,[34] offering tailored solutions for different research needs. Table 1 offers a comprehensive overview, along with a critical analysis of the strengths and weaknesses, of the prevalent microfluidic system archetypes employed for single-cell pairing purposes. For instance, microfluidic droplet systems distinguish themselves as a high-throughput platform that encapsulates individual cells within discrete droplets, guaranteeing minimal cross-contamination and enabling intricate interaction analysis. Undoubtedly, these droplet systems have achieved notable accomplishments in the landscape of genomics research, ushering in advancements that have significantly propelled the field. Nonetheless, their operational precision and the constraint of limited nutrient availability within the droplets pose limitations, restricting their utilization primarily to short-term interaction studies. Thus, researchers must meticulously select the most suitable microfluidic system form based on the specific application requirements. Furthermore, microfluidic platforms integrate with a range of downstream detection technologies, including mass spectrometry,[62] time-lapse microscopies,[63] Raman spectroscopy,[38] and electrophysiological recording.[64] This integration enables the direct, reliable, precise, and accurate evaluation of interaction mediators, allowing researchers to gain deeper insights into single-cell interactions.

In this review, we commence by providing an overarching perspective of the diverse mediators instrumental in cell-cell interaction and communication, delving into their unique characteristics and the innovative detection methods facilitated by microfluidics chips. We then proceed to critically scrutinize microfluidic platforms, meticulously weighing their strengths and weaknesses in enabling the capture of paired single-cells for an in-depth exploration of cellular interactions and communication at the single-cell resolution (Figure 2). Subsequently, we embark on an exploration of the vast array of microfluidic-based applications in the realm of single-cell interaction, encompassing pivotal fields such as immune biology, cancer metastasis, and neurodegenerative diseases. We underscore the potential of these technologies in enhancing the understanding of these complex biological phenomena. Lastly, we conclude by presenting a frank assessment of the current limitations and formidable challenges that lie ahead in advancing single-cell interaction studies utilizing microfluidic tools, which underscores the urgent need for ongoing research and development to overcome these barriers and propel the field toward newly discoveries.

PROFILING A DIVERSE ARRAY OF MEDIATORS UTILIZING MICROFLUIDICS-BASED TOOLS

Mediators that facilitate cell-cell interactions and communication encompass a diverse spectrum, including proteins and peptides (e.g., cytokines, chemokines), nucleic acids (DNA, microRNA, among others), extracellular vesicles, and a myriad of small molecules (hormones, NO, CO, and more), as depicted in Figure 1. Gaining a profound understanding of these intercellular signaling mediators is imperative for unraveling the intricacies of cell-cell interaction and communication mechanisms. To delve into these mediators, cells were initially paired utilizing the aforementioned microfluidic platforms, enabling subsequent analysis through a range of on-chip and off-chip detection techniques. This section delves into the mediators involved in cell-cell interactions,

Table 1. A comprehensive overview and critical analysis of the prevalent forms of microfluidic systems employed for studying single-cell interactions

Strategies	Principles	Advantages	Disadvantages
Microwells and microchambers array[51–53,65]	Randomly inoculate the cell suspension into microwells or microchambers with precision and uniformity.	Relative high through-put; user-friendly operational process.	Experiencing limited capture efficiency, leading to an unnecessary expenditure of manpower and resources that could potentially be optimized.
Droplet-based system[54–56,66–68]	Employing a uniquely designed microchannel architecture that strategically harnesses the shearing force of oil or an incompatible creeping fluid to effectively disperse and mix the cell suspension.	High through-put; manipulate cell components deterministically.	Restricted by limited volume and nutrient availability; may not be ideally suited for the cultivation of adherent cells.
Physical trapping method[44,58,59,69–78]	Capturing cells efficiently within a channel by leveraging either hydrodynamic or dielectrophoretic forces, while also incorporating bypassing channels for optimized flow control and cell confinement.	More precise cell matching; relatively high throughput.	Demanding precise fluid control and physical traps of exact dimensions, while also necessitating the use of external devices and addressing potential issues of cell clogging for reliable operation.
Optical, acoustical and magnetic microfluidic devices[36,60,61,79]	Precisely manipulating and pairing individual cells within microfluidic devices, utilizing non-contact forces such as acoustics, magnetization, and optics, among others, to achieve seamless and controlled interactions.	Minimizing physiological damage to cells; capture of specific single cells from large sample volumes with unparalleled precision and accuracy.	Challenging to simultaneously control heterotypic individual cells; magnetic beads may inadvertently impact cell biology and potentially cause harm to the cells themselves.

expounding on their characteristics and exploring the detection methodologies employing microfluidics-based tools.

Protein measurement utilizing microchip-based technology

Proteins play a pivotal role in orchestrating biological processes, shaping cellular structures, transporting molecules, and catalyzing essential biochemical reactions. Additionally, they act as indispensable signal transducers, mediating the crucial exchange of information between cells. Among these versatile proteins, cytokines emerge as key signaling mediators, produced by immune cells to relay cellular signals via autocrine, paracrine, and endocrine routes. The landscape of protein and peptide quantification is rich with established techniques, including western blotting, mass spectrometry, and enzyme-linked immunosorbent assay, with a particular emphasis on cytokine detection. Innovative approaches like fluorescence-activated cell sorting have even ventured into detecting intracellular proteins, including cytokines within the cytoplasm, by manipulating vesicle transport.[80] Integrating these techniques with single-cell microfluidic platforms promises to unveil mechanisms obscured at the population level. For instance, Fan et al. have showcased an advanced integrated microfluidic system capable of sensitively detecting a broad spectrum of protein biomarkers from minute volumes of whole blood.[81]

Furthermore, certain detection methodologies integrate with microfluidic systems, negating the need for supplementary tools. Lu et al.'s work, which harnessed spatial and spectral encoding alongside microchambers to simultaneously detect 42 proteins secreted by single cells,[18] underscores this potential. This methodology has been further extended to co-detect proteins and extracellular vesicles (EVs).[47] Numerous single-cell functional proteomics tools now offer robust platforms capable of assaying scores of proteins, becoming staples in many research laboratories (Figure 3A).[82–84] For methods that are less compatible with direct microfluidic integration, off-chip detection strategies remain valuable. For instance, transcriptomic analyses can indirectly infer the expression of proteinic signal ligands, serving as a widely adopted approach for protein and peptide quantification.[85]

Nucleic acids function as vital messengers in facilitating cell-cell interactions

In recent years, nucleic acids, comprising DNA, messenger RNAs (mRNAs), and various non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), have emerged as pivotal players in the intricate choreography of information transfer and regulatory control across biological systems.[86,87] These molecules have transcended their traditional role of encoding genetic blueprints, assuming the mantle of communication highways that facilitate the transmission of intricate information between protein binding sites over extensive intracellular distances.[88] Cell-cell communication orchestrated by nucleic acids can exert profound influences on adjacent cells, either directly or through the facilitation of transport mechanisms like extracellular vesicles. This mode of interaction is integral to a wide array of biological processes. miRNAs conveyed via EVs have been shown to directly modulate tumor cell invasiveness and motility, thereby reshaping the tumor microenvironment in critical ways.[89] For instance, Zhang et al. elegantly combined exosomal miRNA analysis with single-cell sequencing technology to uncover a pivotal role of miR-9-5p in

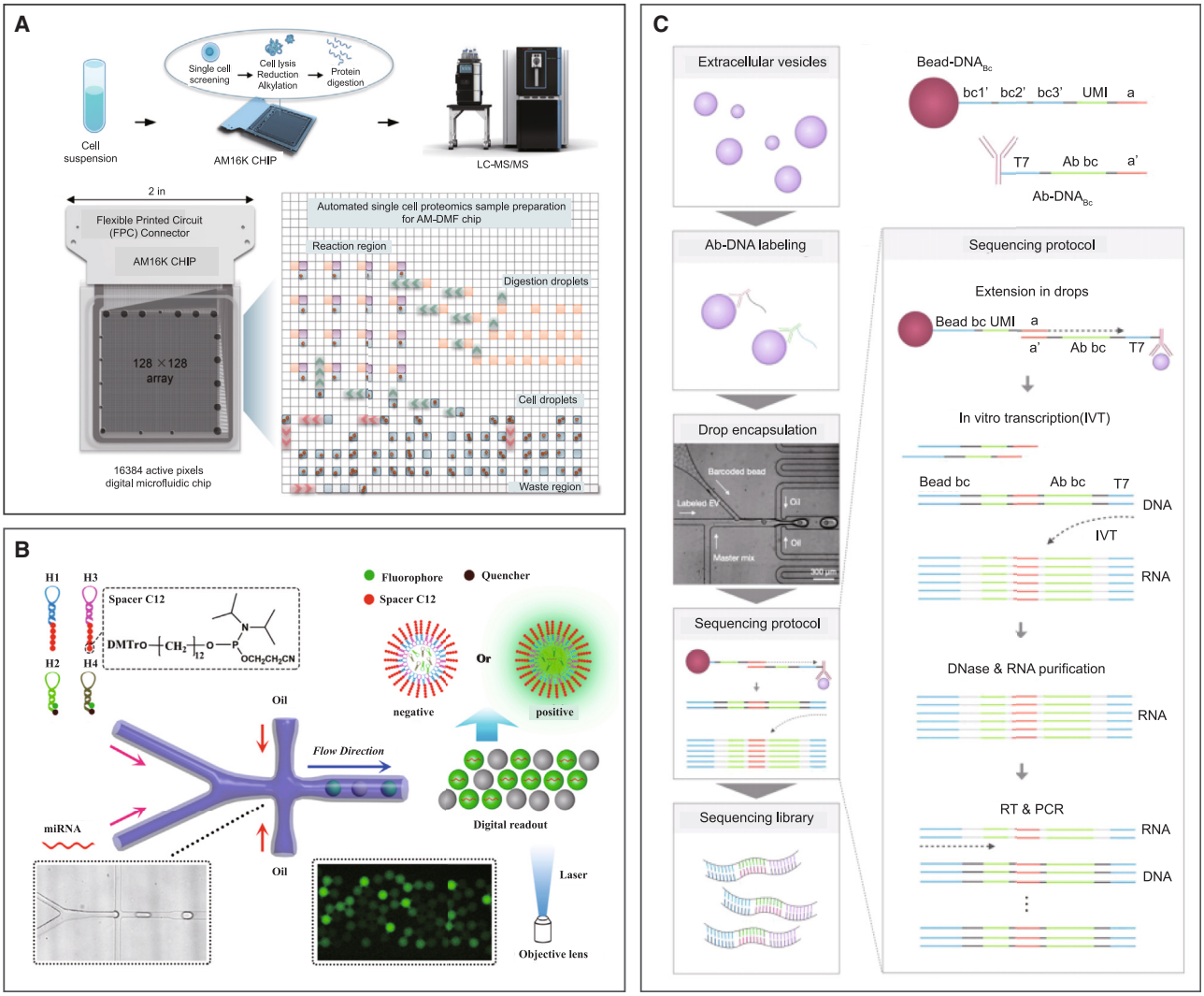

Figure 3. Profiling a wide spectrum of mediators through the utilization of cutting-edge microfluidics-based tools

(A) Single cell proteomics with an active-matrix digital microfluidic platform.[82] (Reproduced from the study by Yang et al.[82] with permission under open license CC BY-NC 4.0, JACS Au.).

(B) Digital quantification of single-molecule microRNA with a digital droplet auto-catalytic hairpin assembly microfluidic system.[92] (Reproduced from the study by Chen et al.[92] with permission from Copyright 2022, Anal Chem.).

(C) Multiplexed measurement of protein molecules from individual nanometer-sized EVs with an antibody-based immunosequencing method.[46] (Reproduced from the study by Ko et al.[46] with permission from Copyright 2021, ACS Nano.).

exosomes.[90] This microRNA regulates the expression of IL-6 in cells, thereby orchestrating a microenvironment that is more conducive to tumor cell proliferation and ultimately accelerating disease progression.

The fundamental principle of complementary base pairing endows DNA and RNA with exceptional capabilities in recognition and assembly, emphasizing the paramount importance of sequencing technologies in unraveling their functional roles. As a result, an array of sophisticated technologies has been leveraged for nucleic acid quantification and analysis, including DNA barcoding techniques that facilitate spatial RNA sequencing. Among these advancements, Liu et al.'s deterministic barcoding method stands out, enabling concurrent mapping of mRNAs and proteins in spatial omics sequencing,[91] marking a significant milestone. To further refine the sensitivity of miRNA detection, Chen et al. ingeniously harnessed microfluidic digital droplet technology to devise an enzyme-free auto-catalytic hairpin assembly system (Figure 3B).[92] This pioneering method facilitates the ultrasensitive quantification of miRNAs, exhibiting an extensive linear range extending from 1 pM to 10 nM, and an exceptional limit of detection of 0.34 pM. This significant improvement is vital for elucidating the intricacies of cell-to-cell communication through miRNAs.

Single-cell nucleic acid detection technology stands out for its high throughput and sensitivity, enabling researchers to uncover subtle cellular differences and decipher nucleic acid-mediated

interactions at the single-cell level. However, microfluidic-based single-cell nucleic acid detection technology encounters numerous limitations and technical hurdles. Efficient isolation and capture of single cells, along with preventing sequencing deviations, are crucial issues that require resolution. Ensuring the accuracy and reliability of sequencing via efficient amplification technology has emerged as a pressing problem that needs to be addressed.

Cell-cell communication mediated by EVs serves as a pivotal mechanism for facilitating interactions between cells

Extracellular vesicles, tiny carriers encapsulated by lipid bilayers, are ubiquitously secreted by virtually all cell types. These vesicles harbor a myriad of biologically active molecules, encompassing proteins, nucleic acids, and lipids, that are crucial for intercellular communication. Extensive research underscores the indispensable role of EVs as messengers in cell-cell interactions.[93,94] For instance, Nikoloff et al. devised a pioneering microfluidic approach that efficiently traps and cultivates single cells, facilitating the immobilization of their secreted extracellular vesicles.[22] This innovative strategy further enables the independent classification of these EVs based on their phenotype, dissociated from their cytosolic origins. The lipid bilayer of EVs safeguards their contents from degradation during transit, while also functioning as a platform for membrane protein receptors to ensure precise delivery to targeted cells.[95] EVs facilitate the transfer of proteins (e.g., membrane proteins, cytokines, growth factors)[96,97] and nucleic acids (RNA, miRNA, lncRNA, and DNA)[98–100] to recipient cells, enabling genetic information exchange and cellular reprogramming. This mode of cell-cell interaction mediated by EVs underpins various physiological and pathological processes.[101] Beyond merely serving as carriers of microRNAs and other molecular cargo, exosomes themselves, notably their surface membrane proteins, occupy a pivotal position in cellular interactions.[102] This cellular heterogeneity profoundly influences EV secretion, with unique EV subtypes intricately intertwined with specific biological processes. Fundamentally, EVs function as versatile intercellular messengers, executing a myriad of biological tasks intimately tied to their intrinsic molecular signatures, thereby emphasizing their critical role in orchestrating intricate cellular communication networks. An illustrative example is the innovative chip developed by Cai et al., which incorporates a polyester thin film filter and a polydimethylsiloxane (PDMS) mesh.[103] Through their analysis, they uncovered a positive correlation between the EV secretion rate of individual glioblastoma cells and the expression level of miR-21 in exosomes, shedding light on the regulatory role of EVs and miR-21 in modulating cell-to-cell communication. To gain a deeper understanding of the complexities of EVs and their functions, researchers have devised an array of innovative approaches aimed at comprehensively characterizing their molecular components, as outlined in recent comprehensive reviews.[104–106]

Profiling EVs from single cells offers a direct avenue to assess the heterogeneity within their parental cell populations. Nevertheless, single-cell resolution EV analysis poses a challenge due to the scarcity and swift diffusion of EVs secreted by individual cells. To overcome this, confining single cells within a delimited environment is crucial, enabling the isolation of secretion profiles from each cell and mitigating diffusion. Furthermore, the nanoliter-scale confinement enhances the relative EV secretome concentration, enabling highly sensitive detection. A diverse array of microfluidic platforms, such as microwells, micromeshes, and microchambers, have been harnessed for single-cell EV assays. For example, Son et al. developed a reconfigurable microwell device to encapsulate single hepatocellular carcinoma cells to realize single-cell EV analysis.[107] Microbeads functionalized with antibodies against CD63 detection antibodies were used for EV capture within a 20 pL micro-compartment. Also, Ji et al. used the spatially patterned antibody barcodes and high throughput microchamber array to realize multiplexed EV secretion with oral squamous cell carcinoma cell lines and primary cells at single-cell resolution.[47] Moreover, microfluidic techniques are developed to reduce the heterogeneity of individual EVs, for example, Ko et al. developed a microfluidic droplet platform for multiplexed profiling surface proteins of EVs based on the antibody immunosequencing method, named single EV immunosequencing (Figure 3C).[46]

Numerous small molecules actively engage in intricate cellular interactions

A diverse array of small molecules plays a pivotal role in cellular communication and interaction by either activating specific enzyme cascades or modulating the cellular environment through direct receptor binding. One prominent example is nitric oxide (NO), which is synthesized from arginine by nitric oxide synthase. NO binds to the Fe-S centers of various enzymes, enhancing the production of cGMP, a versatile messenger molecule that mediates protein phosphorylation and other vital biological processes. Remarkably, NO transmission does not necessitate dedicated receptors, and its fleeting half-life ensures swift diffusion from intracellular to extracellular spaces. Acting as a multifaceted target protein, NO's influence is fleeting, dissipating within seconds. This rapid activity is exemplified by the augmented NO levels observed in vascular tissue construction systems, following the interaction between mesenchymal stem cells and HUVECs.[108] Hormones, another class of small molecules, often peptides or steroids, are produced by regulatory cells and, upon binding to their receptors, alter the physiological functions of target cells, modulating their metabolic processes. Furthermore, an extensive range of small molecules, including hydrogen sulfide (H_2S) and carbon monoxide (CO), participate in intricate cell-to-cell communication networks.[109,110] Despite the crucial role small molecules play in intercellular interactions, the study on detecting small molecule signals using microfluidic single-cell technology is comparatively limited, which is due to the unique characteristics of small molecule signaling media. First, the quantity of small molecules engaging in mutual interactions is minute and highly unstable, exemplified by the rapid reabsorption of dopamine secreted by nerve cells. Consequently, this elevates the technical challenges associated with small molecule detection.

Collectively, cellular interactions are orchestrated by intricate signaling networks, comprising a myriad of mediators. A comprehensive analysis of these signaling profiles would not

only enhance the comprehension of how cells interact within this complex network but also unlock novel biological insights.

OPTIMIZED MICROFLUIDIC PLATFORMS FOR FACILITATING PRECISE AND CONTROLLED INTERACTIONS AND COMMUNICATION BETWEEN PAIRED SINGLE-CELLS

Microfluidics-driven single-cell interaction analysis tools aspire to establish a secluded environment for two or more isolated cells, fostering single-cell level measurements of intercellular interactions. Herein, we introduce an array of microfluidic platforms specifically designed for the intricate analysis of single-cell interactions. This encompasses microwell and microchamber arrays, microdroplets, as well as physical trapping and advanced microfluidic devices leveraging optical, acoustical, and magnetic manipulation. We comprehensively assess each platform's unique strengths, limitations, and the fundamental models upon which they operate.

Microwell and microchamber arrays: Intricately designed structures for isolating and arranging cells within precise spatial confines

Microwell and microchamber arrays have emerged as powerful tools for pairing single cells, harnessing the force of gravity to settle cells randomly within their intricate geometries. A diverse palette of materials is utilized in their fabrication, with PDMS standing out for its biocompatibility, cost-effectiveness, and optical clarity, while agarose gel boasts of its hydrophilicity, nanoscale porosity, exceptional light transmission, and minimal background noise. Although gel-based microwells offer advantages such as nucleic acid electrophoresis and *in situ* decomposition, their inherent mechanical weaknesses limit their applicability in long-term culturing and contact measurements. Navigating through material advancements, the microwell systems still confront the technical hurdle of limited resolution. The Poisson distribution of cell seeding complicates efforts to ensure that each microwell captures a solitary cell pair, albeit high-throughput arrays can compensate by generating substantial quantities of paired cells. Owing to the integration of automation in modern microscopy systems and the sophisticated analysis macros, cell counting and interaction analysis have transformed into streamlined and convenient processes.

For instance, Yamanaka et al. employed arrays of subnanoliter wells to observe individual interactions between NK cells and target cells, and to quantify the subsequent cytolytic and secretory responses.[111] They monitored hundreds of isolated NK cell-target cell interactions and analyzed the correlations among cytolytic activity, secretory activity, and motility. Nonetheless, microwell and microchamber arrays distinguish themselves by their ability to generate a myriad of cell number combinations for direct comparative analysis. Innovative designs facilitate the efficient alignment of cells of varying sizes at high throughput, facilitated by hydrophilic treatments and centrifugation (Figure 4A).[51] The single-cell barcode chip,[112,113] an advanced microchamber array (Figure 4B), encapsulates individual cells or pairs within minute volumes equipped with miniaturized antibody arrays, allowing the selective detection of multiple secreted mediators. By fine-tuning microchannel diameters or cell suspension densities, researchers can precisely tailor the capture efficiency of paired single cells.

However, microwell and microchamber arrays encounter limitations and challenges in the realm of single-cell analysis, notably inefficient cell trapping, variability in cell manipulation, and hurdles in achieving high-throughput analysis. Furthermore, the transition of these systems into clinical applications frequently confronts obstacles related to reproducibility and scalability. Potential strategies for future enhancements may encompass refining microwell designs to bolster cell capture efficiency, augmenting detection sensitivity and resolution, and engineering integrated systems that simplify sample processing and data analysis workflows. Addressing these challenges could herald a new era of more precise and dependable single-cell analysis utilizing microfluidic microwell arrays.

Microdroplets: Minute, encapsulating entities that offer a discrete environment for conducting cell-cell communication

Microdroplets are typically generated by leveraging intricate designs of microchannel structures, which facilitate the segregation of oil or incompatible fluids, often need the assistance of surfactants, into discrete droplets through shear forces.[114] These microchannels encompass diverse geometries such as tee-junctions,[115] fluid focusing,[116] and shaft-like configurations,[117] each tailored to specific applications. Each of these uniform, picoliter-sized droplets acts as an isolated chamber, minimizing cross-contamination and serving as micro-incubators for single cells or cell pairs to thrive in an aqueous environment. This technology has been harnessed by researchers like Antona et al. to quantitatively assess IFN-ɣ secretion from individual natural killer cells in relation to their cytotoxic activity against cancer cells (Figure 5A).[118] Within these droplets, the encapsulation of two or more cell types is meticulously controlled, enabling the exploration of single-cell interactions. The ensuing distribution of encapsulated cell combinations follows the Poisson distribution, resulting in a relatively sparse occurrence and proportion of efficacious droplets.

One significant advantage of droplet microfluidics lies in its remarkable throughput capacity, with droplet generation rates exceeding 1,000 Hz, ensuring statistically robust data. By adjusting the density of the cell suspension, researchers can approximate the number of cells within each droplet, crucial for single-cell interaction studies requiring precise control over cell numbers and ratios. This challenge is further addressed through the integration of additional control techniques, like optics and electricity, as demonstrated by Madrigal et al. who combined dielectrophoresis with deterministic merging to assemble droplets containing defined combinations of cells, beads, and reagents (Figure 5B).[54] Droplets can also be integrated with cutting-edge technologies like 3D printing, enabling deterministic control and precise manipulation (Figure 5C).[119]

However, droplet-based systems have their limitations, particularly in terms of the limited volume and nutrient availability, restricting their application for long-term interaction studies. Moreover, they are not well-suited for adherent cells, which require specific surfaces for attachment and may undergo apoptosis

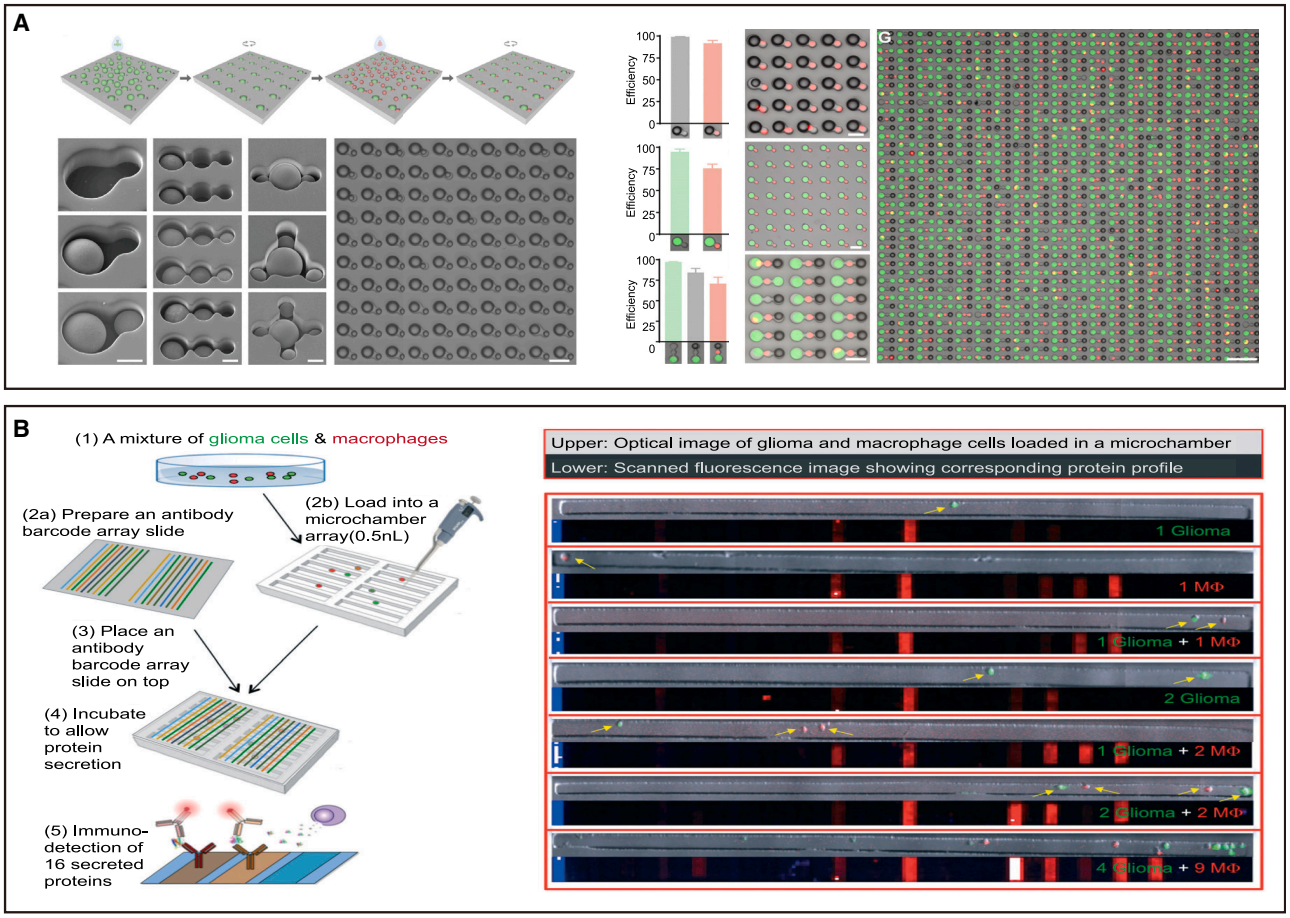

Figure 4. Microwell and microchamber arrays designs for facilitating single cell pairing

(A) A high-throughput hierarchical loading microwell chip, capable of efficiently aligning multiple cells for cell-cell pairing.[51] (Reproduced from the study by Zhou et al.[51] with permission under open license CC BY-NC 4.0, Cell Rep.).

(B) An illustrative representation of a spatial barcoding microchip featuring microchamber arrays for single cell pairing.[113] (Reproduced from the study by Elitas et al.[113] with permission from Copyright 2014, Lab Chip.).

in a droplet environment. Innovations like Kim et al.'s approach, which encapsulates cells within GelMa hydrogel bioink and alginate matrices, offer a promising solution by transitioning the cells from 2D to 3D cultures, enhancing cell-to-cell interactions akin to those observed in cell spheroids.[55] This advancement underscores the continued evolution of droplet technology toward more sophisticated and adaptable systems for cell biology research.

Refined physical trapping technique

The physical trapping method, an oft-utilized technique for pairing single cells, relies heavily on cell size and deformability. It involves directing cells toward predefined traps via hydrodynamic or dielectrophoretic forces within a channel network that incorporates bypass channels. This allows for the delivery of cells to traps in varying directions, facilitating diverse single-cell pairing configurations. Once captured, the cells face difficulties in escaping, facilitating their contact and communication. In contrast to the random pairing seen in microwell and droplet-based methods, the physical trapping technique offers a heightened degree of precision in cell matching, with pairing efficiencies reaching 70–80% and more.[120] For instance, Frimat et al. presented a highly parallel microfluidic approach utilizing differential fluidic resistance trapping, enabling the efficient pairing of single cells. Their method achieved a remarkable 99% efficiency in cell arraying and supported long-term cell culture, thereby facilitating an impressive 70% rate of cell pairing (Figure 6A).[121] Dura et al. introduce a high-throughput microfluidic platform capable of deterministic pairing of lymphocytes with remarkable efficacy (Figure 6B). This innovative platform captures up to 80% of cells entering the array, achieving cell pairing efficiencies spanning from 40 to 85%.[122] Furthermore, physical trapping enables pairing not just horizontally but also vertically, as exemplified by Jang et al.'s double-layered chip designed for investigating immune-target cell interactions.[123] For pairing cells of disparate sizes, Shaik et al. innovated by modulating channel height in the z-direction through hydrodynamic flow focusing, achieving size-specific single-cell pairing (Figure 6C).[57]

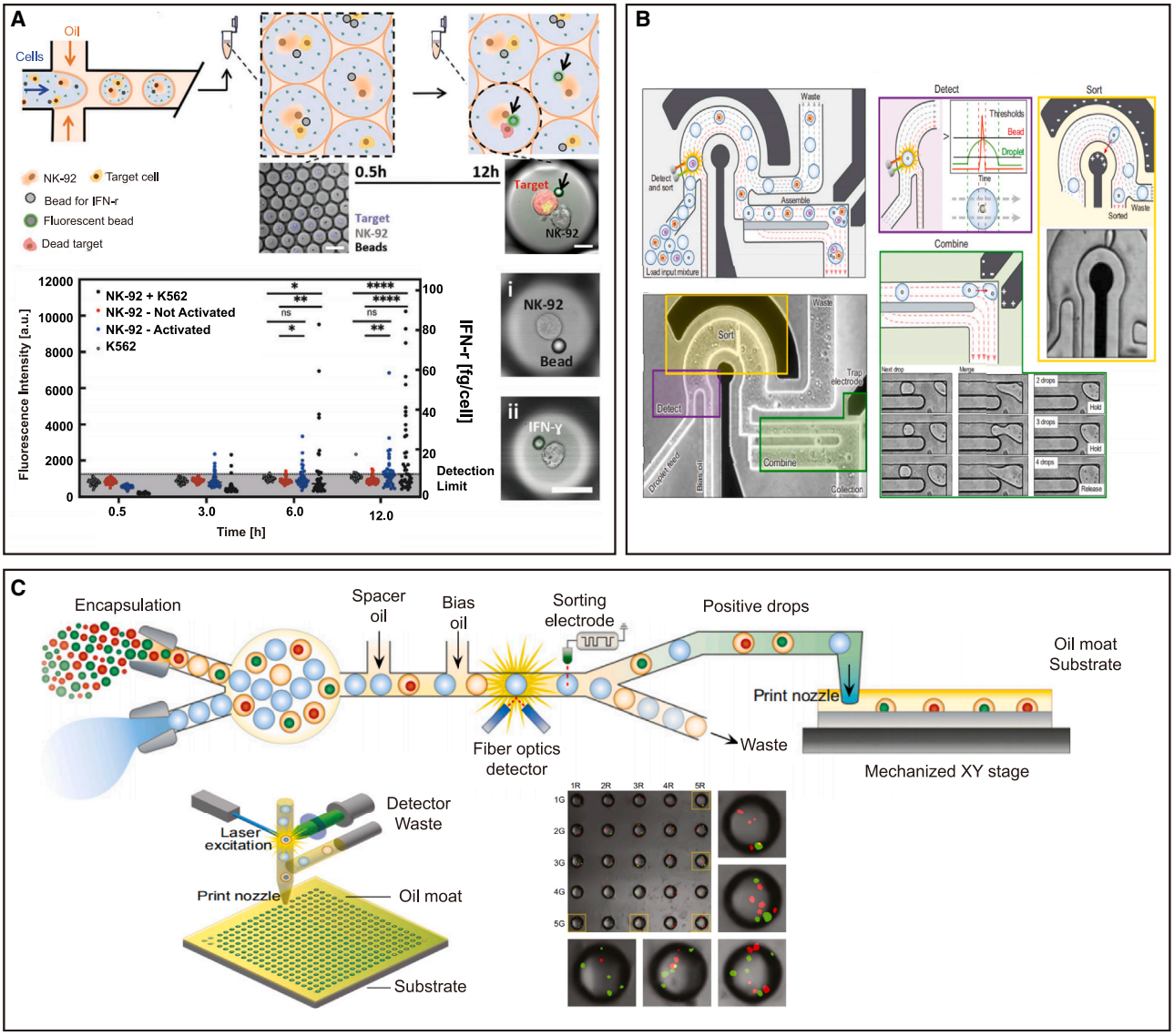

Figure 5. Microdroplets for conducting cell-cell communication

(A) A droplet-based microfluidic technique for pairing individual cells.[118] (Reproduced from the study by Antona et al.[118] with permission under open license CC BY-NC 4.0, Adv Funct Mater.).

(B) A high-throughput droplet microfluidic system that deterministically constructs multicellular combinations for investigating cell combinations and interactions.[54] (Reproduced from the study by Madrigal et al.[54] with permission under open license CC BY-NC 4.0, Proc Natl Acad Sci USA.).

(C) A printed droplet microfluidic platform that precisely prints droplets containing reagents and cells into defined arrays in a deterministic fashion.[119] (Reproduced from the study by Madrigal et al.[119] with permission under open license CC BY-NC 4.0, Proc Natl Acad Sci USA.).

One of the primary challenges in achieving one-to-one single-cell pairing with physical traps lies in the meticulous control of fluid dynamics. Inaccuracies in fluid flow can lead to cell loss from traps, also affecting cell properties. Therefore, precise trap design is paramount, as it ensures the capture of only a single cell pair, enhancing accuracy. Additionally, flow channel design must be optimized to avoid low capture rates or confined cultivation spaces, which could compromise results.

To further enhance pairing efficiency, throughput and traceability, researchers have explored the integration of multiple single-cell pairing strategies. Fan et al. illustrated this by combining hydrodynamic and recirculation flows to achieve high-throughput and deterministic single-cell pairing in microwell arrays, enabling traceable coculture. They reported remarkable pairing efficiencies of 72.2% and 38.0% for double and triple pairings, respectively.[58] However, when dealing with large cell numbers, cell clogging becomes a concern. To address this and cater to different fluidic requirements, researchers utilize a diverse range of materials for chip fabrication, including PDMS, ITO glass, and SU8 photoresist.

Optical, acoustical and magnetic microfluidic devices

In addition to the passive techniques mentioned earlier for achieving cell pairing, microfluidic devices offer active methods for manipulating and pairing single cells using non-contact forces, such as optics, acoustics, and magnetism.[43,61] Optical tweezers, renowned for their exceptional precision (reaching nanometer levels, down to 10 nm), operate without physical contact and integrate into microfluidic platforms for single-cell investigations. Jing et al. innovated a optical tweezers system, facilitating studies on cell adhesion and organization, enabling precise cell patterning and cultivation (Figure 7A).[61] However, these optical microfluidic systems face operational complexities and high equipment costs, constraining their widespread adoption. Furthermore, their reliance on a transparent medium and potential for cell damage due to heat generation are notable limitations.

Surface acoustic waves (SAW) have emerged as a powerful tool for precise manipulation of both individual cells and fluids, enabling the study of cell-cell interactions. Guo et al. demonstrated SAW's ability to control the distance and spatial organization of single cells, visualizing fluorescent dye transfer through gap junctions to investigate intercellular communication (Figure 7B).[79] Acoustofluidics boasts minimal physiological damage to cells during manipulation and easy integration with standard microfluidic systems. Nevertheless, simultaneously controlling heterotypic cells remains a challenge with SAW.

Magnetic manipulation of single cells, achieved by attaching cells to magnetic beads, presents a unique approach for studying single-cell interactions. Lim et al. leveraged this technique to position single T and B cell pairs in predetermined trajectories for downstream analyses (Figure 7C).[124] While magnetic manipulation does not require direct cell contact, the use of magnetic beads may influence cell properties and subsequent studies. Nevertheless, the magnetic field's extensive coverage allows for the efficient capture of specific single cells from large sample volumes, offering a distinct advantage over other single-cell manipulation technologies.

To summarize briefly, each microfluidics-driven single-cell interaction analysis tool exhibits unique advantages and disadvantages, influencing their applicability in specific scenarios. Microwell and microchamber arrays offer user-friendly operational process but may suffer from limited capture efficiency. Microdroplets excel in high-throughput single-cell interaction analysis but face challenges in cell viability and droplet fusion. Physical trapping methods are straightforward but may limit cell manipulation flexibility while suffering clogging. Optical techniques provide non-contact manipulation with high precision but require sophisticated equipment. Acoustic devices enable gentle cell handling but can be limited by frequency-dependent effects. Magnetic systems offer scalability and remote control but require cell labeling, which may affect cell function. Choosing the right microfluidic form depends on the balance between precision, throughput, cell viability, and system complexity, tailored to the specific research or clinical needs.

INSIGHTS INTO NOVEL BIOLOGY GARNERED THROUGH THE STUDY OF MICROFLUIDICS-BASED SINGLE-CELL INTERACTIONS AND COMMUNICATION

Microfluidics-based technologies for studying single-cell interactions have gained widespread adoption in domains such as immune monitoring, tumor research, and neural development. These advancements allow researchers to monitor cellular functional heterogeneity, trace dynamic immune responses, and investigate intercellular signal communication. This precision unlocks a myriad of novel biological insights that have hitherto remained unobserved. Table 2 presents select illustrative examples of intercellular communication that have been scrutinized by employing microfluidic strategies to detect intercellular signal mediators at the single-cell level. In the subsequent section, we delve into applications that pose significant challenges for traditional population-level cell analysis methods across various biological disciplines, including immunology, oncology, and neurology, but are well-suited for exploration using microfluidic-based single-cell interaction technologies.

Immunology

The intricate interplay between immune cells constructs a vital immunological network essential for upholding human homeostasis and safeguarding against invading pathogens and tissue injury. Imbalances within this intricate network frequently precipitate either exaggerated or absent immune responses, ultimately predisposing individuals to a range of diseases. Given the remarkable heterogeneity inherent in immune cells, scrutinizing their interactions at the single-cell level has become paramount for enhancing immunological assessments.

Microfluidic technology, with its prowess in crafting precisely tailored microenvironments endowed with spatiotemporal control via device geometry, surface chemistry, and fluidic dynamics, has emerged as a potent tool for unraveling the complexities of immune systems. Dura et al., for instance, harnessed a microfluidic chip integrating a groove with a U-shaped trap for parallel, single-cell level analysis of lymphocyte interactions, achieving over 50% pairing efficiency through a four-step loading technique.[128] This innovative approach illuminated the kinetic activity of T cells upon activation, a phenomenon difficult to capture using traditional methodologies. Furthermore, researchers have delved into the cross-talk between immune and non-immune cells. Tu et al. devised a microfluidic microwell array facilitating massive parallel examination of immunocyte heterogeneity and its dynamic interactions with tumor cells at the single-cell resolution.[53] Their investigation unveiled time-dependent killing dynamics and drug-induced shifts, insights obscured in bulk-level studies. Additionally, studies on NK-tumor cell interactions have directly correlated quantitative variations in NK cell calcium signals with their functional outputs, enriching the understanding (Figure 8A).[122] Ide et al. introduced an open-type PDMS microfluidic device, a physical trapping platform, to probe the interplay between T cells and antigen-presenting cells, pioneering the quantification of the T cell activation threshold.[70] Huuhtanen et al. leveraged single-cell RNA sequencing and T cell receptor profiling to reveal heightened communication between T-LGLL clonotypes and non-leukemic immune cells compared to reactive clones.[129] Wang et al., meanwhile, developed a microfluidic method to encapsulate and culture individual fibroblast and T cells within a hydrogel matrix, enabling assessments of how spatial constraints, hydrogel structure, and mechanics modulate cell behavior and influence drug responses through cell-extracellular matrix interactions.[130]

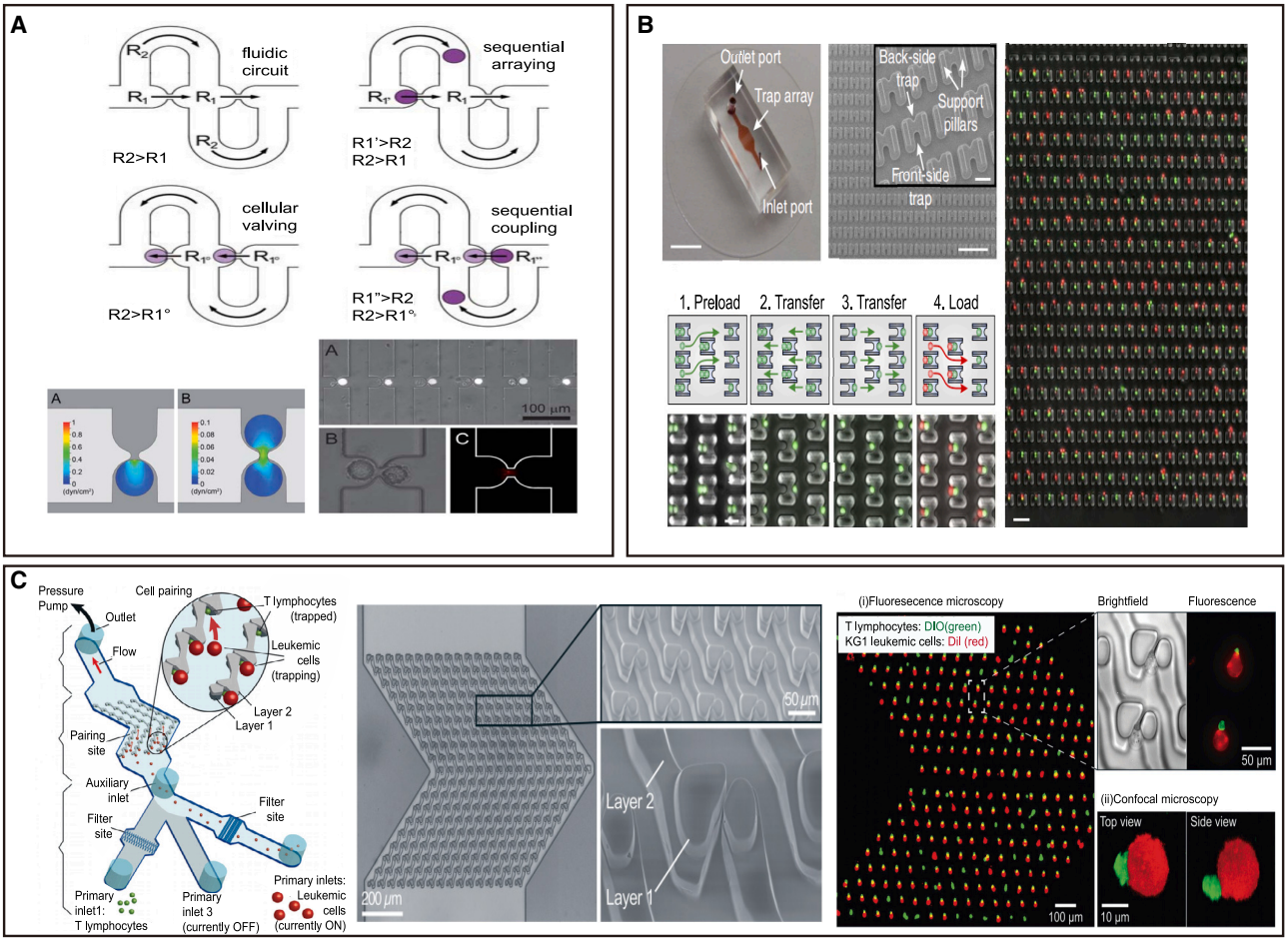

Figure 6. Physical Trapping based microfluidics devices for conducting cell-cell communication

(A) A highly parallel microfluidic system employing fluidic resistance trapping to facilitate contact between individual cell pairs.[121] (Reproduced from the study by Frimat et al.[121] with permission from Copyright 2011, Lab Chip.).

(B) A microfluidic co-culture platform utilizing hydrodynamic traps developed for studying immunology and the implications of cell-cell interactions.[122] (Reproduced from the study by Dura et al.[122] with permission from Copyright 2015, Nat Commun.).

(C) Physical trapping microfluidic devices, which harness z-direction hydrodynamic flow focusing, enable the capture of cell pairs with enhanced precision.[57] (Reproduced from the study by Shaik et al.[57] with permission under open license CC BY-NC 3.0, Lab Chip.).

Collectively, these findings underscore the immense potential of microfluidic-based single-cell interaction studies in advancing immunological research.

Oncology

Cancer, a formidable adversary to human health, accounts for a staggering quarter of all disease-related deaths. The tumor microenvironment, a complex ecosystem of neighboring tumor cells, diverse stromal elements including cancer-associated fibroblasts (CAFs), immune cells, bacteria, and dynamically evolving bioinformatic molecular networks, serves as the cradle for cancer cell survival and functional execution. The intricate interplay between cancer and stromal cells orchestrates critical processes such as tumor proliferation, migration, invasion, angiogenesis, and eventual distant metastasis. Illustratively, Jobe et al. discovered that interleukin-6 (IL-6) secreted by CAFs fuels melanoma's invasion into the surrounding type I collagen matrix.[131] Moreover, this interaction triggers CAFs to release IL-8, and modulating the production of these cytokines through drug interventions has emerged as a strategy to inhibit tumor metastasis. Jian et al. further highlighted that elevated IL-6 levels in CAFs of colon cancer stimulate the secretion of vascular endothelial growth factor, thereby promoting tumor angiogenesis.[132] However, traditional population-based assays often obscure the heterogeneity in tumor-stromal interactions, which may underlie drug resistance and tumor immune evasion in certain cancers. Microfluidic technology, through its capability for single-cell interaction analysis, holds the potential to fundamentally address this challenge.

Given the critical modulation of immune cells within the tumor microenvironment by cancer cells, targeting the immune response has emerged as a promising therapeutic avenue. Recent studies have leveraged microfluidic chips to investigate immune-tumor interactions. Li and colleagues, for instance, devised a

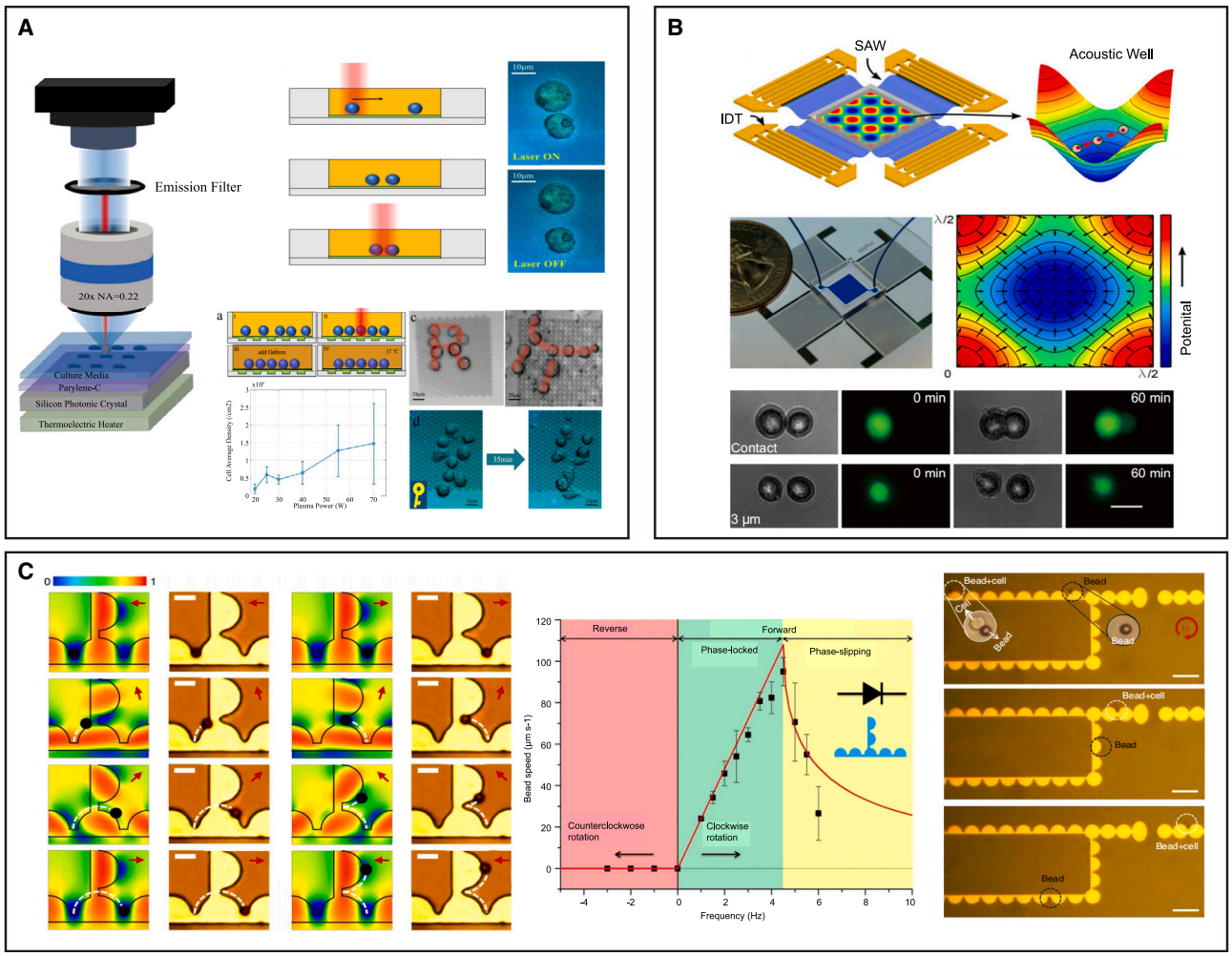

Figure 7. Advanced optical, acoustical, and magnetic based microfluidic devices for studying cell pairing and cell-cell communication

(A) Optical Tweezers, a approach for precisely pairing individual cells.[61] (Reproduced from the study by Jing et al.[61] with permission under open license CC BY-NC 4.0, Biomed Opt Express.).

(B) An innovative acoustofluidic method for single cell pairing.[79] (Reproduced from the study by Guo et al.[79] with permission under open license CC BY-NC 4.0, Proc Natl Acad Sci USA.).

(C) Scalable integrated circuits designed to transport magnetic particles and single cells along programmable routes within microfluidic setups.[124] (Reproduced from the study by Lim et al.[124] with permission under open license CC BY 3.0, Nat Commun.).

centrifugal-based hydrodynamic microfluidic chip that enables the study of long-term interactions between neutrophils and cancer cells (Figure 8B).[74] Their findings underscore the differential impact of HeLa cell lines on neutrophil migration, with interleukin-8 attracting neutrophils while IL-10 eliciting opposing migratory effects. Furthermore, the tumor microenvironment significantly influences cancer progression and outcomes. Li et al. developed a single-cell analysis platform that integrates both physical and physiological cues to study single-cell interactions. Notably, they demonstrated that macrophages in contact with cancer cells undergo polarization from the M1 to M2 phenotype within this engineered tumor microenvironment, offering insights into the dynamic interplay between immune cells and cancer in shaping tumor fate.[133] As another example, Nikoloff et al. conducted an analysis of the protein profiles of individual extracellular vesicles derived from single breast cancer cells, a task that is inherently challenging without the aid of microfluidics technology.[22] Their findings underscore the remarkable informativeness of the phenotypic examination of EVs originating from a single cell, sufficient to reveal distinct differences in their origins.

Neurology

The intricate architecture of the human nervous system encompasses the central and peripheral nervous systems, with neurons serving as the pivotal functional units. These neuronal cells engage in intricate interactions with their neighboring cells, including infiltrating and resident immune cells, to collaboratively execute neural functions. A profound comprehension of these single-cell interactions holds the key to unlocking novel insights into brain development, as well as the onset and progression of

Table 2. A comprehensive overview of the prevalent biological and clinical applications that harness the power of microfluidic-based single-cell interaction technology

Forms of systems	Diverse mediators	Technical characteristics	Applications and biological insights
Microwell	Cytokines	Efficiently aligning multiple cells and functionalized beads within a high-throughput microwell array, leveraging their size differences for precise positioning.	Integrating measurements of cytotoxicity and cytokine secretion from individual lymphocytes, and they elucidated the intricate linkages between their functional capabilities and cellular behaviors.[51]
	protein	Integrating spatially patterned antibody barcodes with microchamber arrays.	Elucidating 16 distinct protein profiles resulting from the intricate interactions between individual glioma cells and single macrophage cells.[113]
	Multi-mediators	Channels (inlets) integrated within a microwell array to provide assisted and efficient fluidic handling and cell positioning.	Unveiling the heterogeneity in the killing capacity of T cells, revealing intricate variations in their effector functions.[53]
	Multi-mediators	Integrating hydrodynamic forces with recirculation flow captures for achieving deterministic and precise pairing of single cells within microwells.	Employing single-cell pairing and coculture methodologies to delved into the intricate mechanisms of cellular engulfment.[58]
	Multi-mediators	Utilizing a microwell array for the generation of neurospheres with rigorously controlled sizes.	The crosstalk between endothelial cells and neurospheres potentiated their self-renewal abilities and stemness characteristics, further promoting the differentiation into astrocytes.[125]
	Cytokines, neurotrophic factors, EVs	Integrating microwell technology with spatially patterned antibody barcodes on a microchip.	Mapping the secretomes from paired neuron-immune single-cells, uncovering the intricate, secretion-mediated interactions that occur between these cells.[126]
Droplet	Multi-mediators	Employing fluorescence-activated droplet screening and sorting, followed by single-cell RNA sequencing (scRNA-seq).	Identifying T cell receptors capable of recognizing peptide-MHC (pMHC) complexes and subsequently analyzed the signaling cascades emanating from these receptors to downstream pathways.[56]
	Cytokines	Integrating dielectrophoretic droplet sorting with deterministic merging, constructed droplets containing precisely defined combinations of components.	They enriched CAR-T cells that were activated upon incubation with cancer cells, and subsequently identified the associated changes in gene expression.[54]
	Interferon gamma (protein)	Droplets containing distinct markers for precise identification and characterization.	They delved into the intricate relationship between the dose of human recombinant IFN-γ and the cytolytic activity exhibited by NK cells.[118]
Physical trap array	Multi-mediators	Utilizing z-direction hydrodynamic flow to capture and position cell pairs with precision and efficiency.	They observed the induced calcium responses in T lymphocytes at the immunological synapse formed between paired living cells, elucidating the intricacies of intercellular communication.[69]
	Multi-mediators	An innovative open-type PDMS microfluidic device was meticulously designed, incorporating the use of hydrodynamic force for enhanced fluid handling and cellular manipulation.	At the single-cell level, they pinpointed the response of T cells, uncovering the intricate heterogeneity that exists among T cell responses.[70]
	ATP (small molecules)	Orchestrating the arrangement of diverse cell types into precise clusters within a flow chamber, tailored for seamless integration with high-resolution microscopy techniques.	They deciphered the intricate relationship between distance and the dynamics of cytosolic calcium changes, shedding light on cellular signaling mechanisms.[120]
	IL-8 and IL-10 (protein)	Combining hydrodynamic single-cell capture with centrifugation-assisted relocation for precise positioning and manipulation of individual cells.	They discovered that HeLa cells exert varying influences on migration processes, underscoring their complexity and multifaceted roles.[74]
	Multi-mediators	Employing biomaterial patterning techniques for the long-term maintenance and viability of single cells.	They established compartmentalized homotypic neuronal cocultures, facilitating the study of neuronal interactions and networks in a physiologically relevant context.[127]

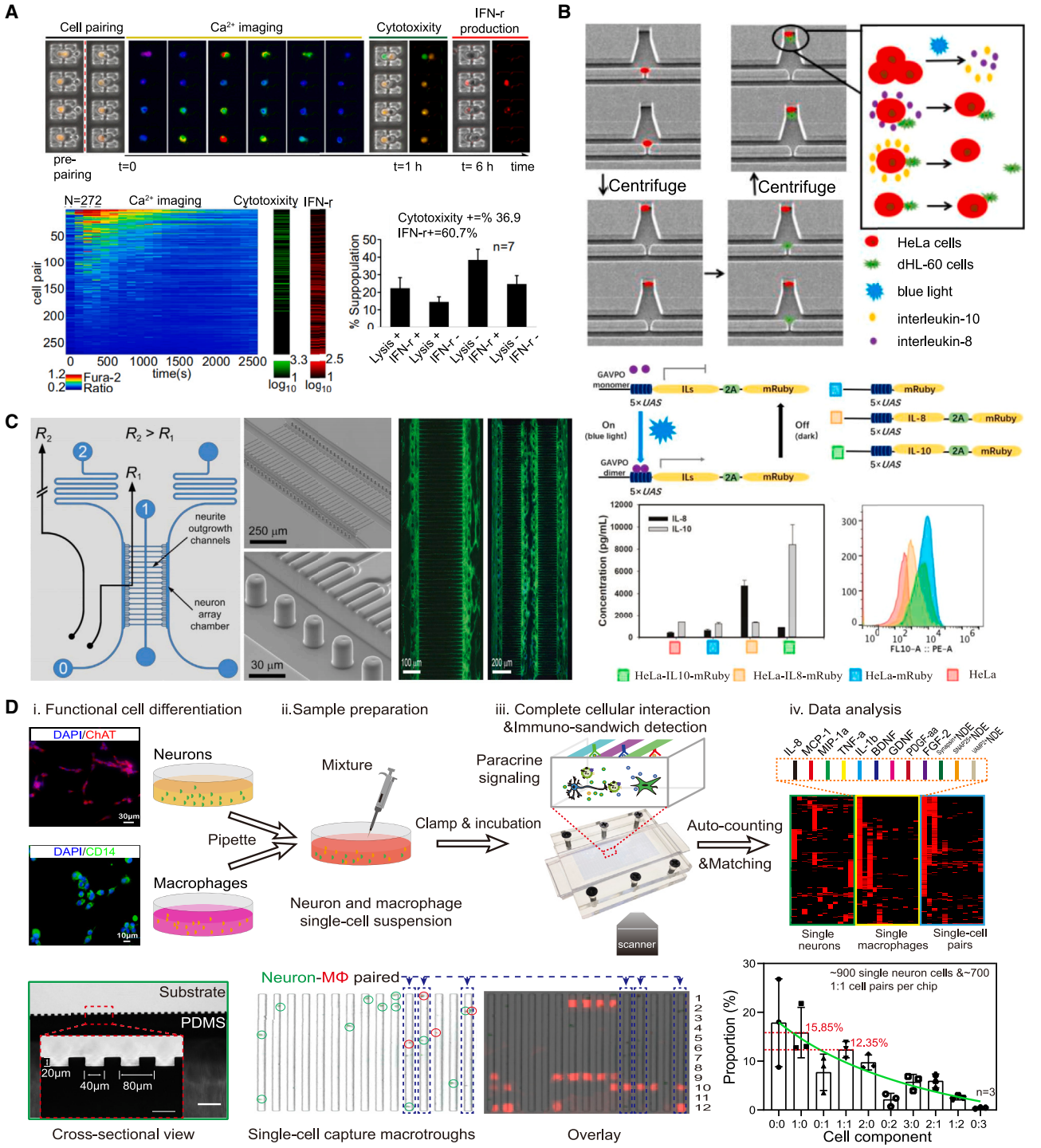

Figure 8. Illustrative applications of microfluidic-based single-cell interaction technologies have advanced the understanding of new biological insights

(A) A detailed examination was conducted into the direct correlation between quantitative differences in calcium signals within NK cells and their subsequent functional outcomes, through the study of NK cell interactions with tumor cells at the single-cell level.[128] (Reproduced from the study by Dura et al.[128] with permission under open license CC BY-NC 4.0, Proc Natl Acad Sci USA.).

(B) An investigation into single-cell interactions between neutrophils and various HeLa cell types revealed that the IL-10 factor secreted by HeLa cells repels dHL-60 cells, whereas IL-8 elicits contrasting effects.[74] (Reproduced from the study by Li et al.[74] with permission from Copyright 2019, Anal Chem.).

(legend continued on next page)

neurodegenerative disorders. Dinh et al. pioneered an approach by establishing compartmentalized homotypic neuronal co-cultures for individual neurons (Figure 8C).[127] Their study not only validated the feasibility of arranging single neurons in arrays but also revealed that clustering neurons through cell-cell contacts accelerated neurite outgrowth. Additionally, microfluidic-based single-cell analysis methods have emerged as powerful tools for investigating neurodevelopment. They further demonstrated this by observing neurite outgrowth utilizing differential fluidic resistances in chambers separated by neurite outgrowth channels, enabling precise single-cell arrays and revealing synaptic tubercles as evidence of neuronal interaction and communication.

In a separate endeavor, Deng et al. leveraged a high-throughput micro-chamber chip equipped with spatially patterned antibody barcodes, an advancement from the single-cell barcode chip technology, to delve into the secretome-mediated neuron-macrophage interaction at the single-cell level (Figure 8D).[126] This innovative platform enabled the simultaneous mapping of 12 diverse secretomes, encompassing cytokines, neurotrophic factors, and neuron-derived exosomes, from paired single cells under both normal and pathological conditions. Their findings underscored significant differences in the cellular interactions between neuron-macrophage and neuron-microglia pairs. Notably, neuron-macrophage interactions triggered immune responses and reduced neuronal secretion, whereas neuron-microglia interactions had opposing effects on secretion, significantly enhancing the understanding of the intricate secretome network that governs neuron-immune cell interactions.

CHALLENGES AND OUTLOOKS

It is widely acknowledged that the intricacies of cell and interaction heterogeneity, alongside the specific interplay between distinct cells within cellular populations, hold a pivotal role in human biology. The advent of microfluidic-based single-cell interaction analysis has significantly progressed, leveraging its advantages of miniaturization, high throughput, and real-time monitoring capabilities in exploring cell-cell interactions. This approach offers vast potential for applications, but not limited, in immunology, oncology, and neurology, shedding light on biological nuances that might remain obscured through traditional methodologies. Nevertheless, several challenges persist and necessitate further attention.

First, the microfluidic platform's capacity to comprehensively replicate *in vivo* cellular interactions is inherently limited, given its potential inability to perfectly emulate the intricate microenvironment of living organisms. To address this limitation and enhance the biological relevance and insights derived from these studies, the incorporation of more advanced bio-mimetic models and technologies, such as organoids or organ-on-a-chip systems, within the microfluidic single-cell interaction framework is paramount. Furthermore, the prevalent single-cell pairing designs often impose restrictive constraints on cellular activities and functionalities, limiting their application to merely short-term interaction analyses. Consequently, it is imperative to prioritize the development of more sophisticated, bio-mimetic single-cell pairing platforms, particularly those that incorporate controllable 3D cell pairing chips. This advancement would not only alleviate these constraints but also facilitate a more comprehensive understanding of cellular dynamics. An optimal approach to detecting cell interactions in a more authentic context is to leverage innovative techniques that allow for *in situ* investigations. One notable example is the pioneering proposal by Feng et al., which showcases an open microfluidic probe specifically tailored for the direct, *in situ* exploration of single-cell communication.[134] This methodology presents a promising avenue for capturing the essence of cellular interactions in their native environments, thereby advancing the knowledge of biological processes.

Second, the focus of cell-cell communication studies on mediators remains narrow, predominantly limited to a select handful of pivotal signals observed at defined endpoints. This constrained analysis of signaling mediators undermines the allure of current technology, as alternative platforms promise to delve into significantly greater depths and provide unparalleled insights. Given the intricacies of signaling pathways between individual cells, isolating individual signals presents a formidable challenge. Integrating a single-cell signaling mediator analysis platform with complementary, high-depth single-cell analysis methods, such as single-cell RNA-seq, to concurrently examine the same samples using a multi-omics approach represents a pivotal and exhilarating direction for advancing the understanding. This interdisciplinary strategy promises to reveal intricate cellular interactions and mechanisms in unprecedented detail.

Third, current research often confines itself to the narrow scope of interactions between just two or three cell types, predicated on the assumption that cellular interactions remain static. This limited perspective fails to fully explore the complex interplay within heterogeneous cell cultures and their dynamic nature. In reality, interacting cells are dynamic entities, encompassing a diverse spectrum of cell types and undergoing temporal variations. By expanding the breadth of investigations to include a diverse array of heteromorphic cells displaying time-varying interactions, researchers can achieve a more precise imitation of the transient and dynamic pairings encountered naturally within living systems. This approach has the potential to substantially enhance the understanding of the intricate heterogeneity mechanisms that underpin single-cell interactions.

Fourth, an exhilarating prospect emerges from the remarkable progress made in integrating cell culture, sorting, transfection, and dissolution detection onto microchips. However, the practical realization of a solitary chip that flawlessly unites all these

(C) The study of interconnected single neuron networks underscores a fundamental principle that neurons organize into clusters, facilitated by cell-cell contacts, to promote the development of neural outgrowths.[127] (Reproduced from the study by Dinh et al.[127] with permission from Copyright 2013, Lab Chip.).
(D) By analyzing the secretome-mediated interactions between paired neuron-macrophage single cells, researchers observed that these pairwise interactions stimulate immune responses and diminish neuronal secretion. In contrast, neuron-microglia interactions typically yield contrasting secretory outcomes.[126] (Reproduced from the study by Deng et al.[126] with permission under open license CC BY-NC 4.0, Proc Natl Acad Sci USA.).

technologies is still a theoretical aspiration, offering a captivating benchmark to strive for and potentially ignite a technological revolution.

Fifth, the vast majority of contemporary single-cell analysis chips, whether they employ microporous arrays or droplet-based technologies, predominantly rely on PDMS materials. However, PDMS's inherent hydrophobicity poses a challenge, as hydrophilic signaling mediators tend to adhere readily to the chip's surface, ultimately hindering the accuracy of experimental results analysis. To surpass these limitations, advancements in chip materials are paramount, addressing the widespread yet restrictive use of PDMS due to its complexities in surface chemical modification and scalability constraints. Consequently, the exploration of innovative materials, including polycarbonate and polystyrene, as potential alternatives to PDMS in microfluidic chip design holds significant promise for advancing this field.

Anyway, there are also many technical difficulties, such as precise cell isolation, maintaining cell viability during processing, and addressing droplet size variability, which can affect sorting accuracy. The high initial investment and maintenance expenses limit the widespread adoption, and clinical translation faces hurdles like ensuring data reproducibility, addressing regulatory concerns, and validating findings in large-scale trials. Despite its potential, overcoming these barriers is crucial for microfluidic technology to become a routine tool in single-cell analysis and clinical practice. However, we anticipate that this field will undergo rapid expansion and find widespread application in biology and medicine as new fabrication techniques and materials continue to evolve. Moreover, with the progression of micro- and nanofabrication technology, we expect the emergence of increasingly accurate and bio-mimetic single-cell interaction platforms, offering unprecedented opportunities for new biological insights.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.Yuan. and J.D.; methodology, P.Z. and J.D.; writing – original draft, J.D.; writing – review and editing, S.Yuan., P.Z., F.Z., R.D., S.Yan., C.W., and J.D.; visualization, S.Yan. and J.D.; supervision, C.W. and J.D.

DECLARATION OF INTERESTS

There are no conflicts to declare.

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