

Microfluidic techniques for isolation, formation, and characterization of circulating tumor cells and clusters



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ABSTRACT

Circulating tumor cell (CTC) clusters that are shed from the primary tumor into the bloodstream are associated with a poor prognosis, elevated metastatic potential, higher proliferation rate, and distinct molecular features compared to single CTCs. Studying CTC clusters may give us information on the differences in the genetic profiles, somatic mutations, and epigenetic changes in circulating cells compared to the primary tumor and metastatic sites. Microfluidic systems offer the means of studying CTC clusters through the ability to efficiently isolate these rare cells from the whole blood of patients in a liquid biopsy. Microfluidics can also be used to develop *in vitro* models of CTC clusters and make possible their characterization and analysis. Ultimately, microfluidic systems can offer the means to gather insight on the complexities of the metastatic process, the biology of cancer, and the potential for developing novel or personalized therapies. In this review, we aim to discuss the advantages and challenges of the existing microfluidic systems for working with CTC clusters. We hope that an improved understanding of the role microfluidics can play in isolation, formation, and characterization of CTC clusters, which can lead to increased sophistication of microfluidic platforms in cancer research.

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I. INTRODUCTION

Circulating tumor cells (CTCs) and their clusters disseminate to distant sites in the body through the bloodstream as part of the metastatic process. Their occurrence is extremely rare, often <100 CTCs or <10 clusters per 10×10^6 leukocytes and 5×10^9 erythrocytes in 1 ml whole blood.^{1–5} Nevertheless, their occurrence far exceeds the number of actual metastatic lesions in patients, supporting the notion that very few CTCs overcome the harsh vascular environment and successfully metastasize onto a secondary site.² Clinical studies have indicated that CTC clusters have prognostic value associated with predicting treatment resistance and survival outcomes,^{6–8} suggesting their importance in developing personalized and novel cancer therapies. These cells can offer information on differences in the genetic profiles, somatic mutations, and epigenetic changes compared to cells in primary tumor and metastatic sites.^{9,10} Yet, detection of CTC clusters has been associated with worse clinical outcomes and higher (estimated as 20–50 fold greater) metastatic potential.² The mechanisms that lead CTCs to acquire an invasive phenotype are yet to be fully understood, and due to the inherent rarity of CTCs and CTC clusters, extensive characterization is limited.

CTCs and clusters are typically isolated directly from blood using immunoselection or flow cytometry, but microfluidic isolation is an attractive alternative only requiring smaller volumes of samples and less procedural steps suitable for diagnosis, molecular analysis, and *in vitro* studies. Microfluidic systems have proven capability in isolation, formation, and characterization of individual cells,¹¹ spheroids,^{12–14} and organoids^{15–17} over the past few years (Fig. 1). Microfluidic methods of isolation and analysis have become more diverse with both physical and biomarker-based techniques to capture, label, and phenotype CTCs. These techniques include the physical and biomarker capture of primary CTCs,¹⁸ induction of physical parameters to assess phenotype,¹⁹ deformability assays,²⁰ protein quantification,²¹ drug response,²² and many others.

In addition, *in vitro* modeling of metastasis is possible by fine spatiotemporal control over a cellular microenvironment. Microfluidics has also been used to study the effects of flow and shear stress on CTCs with the use of modified channel geometries, flow rates, and reagent compositions. With limited sample quantities, further characterization may require the *in vitro* formation of clusters

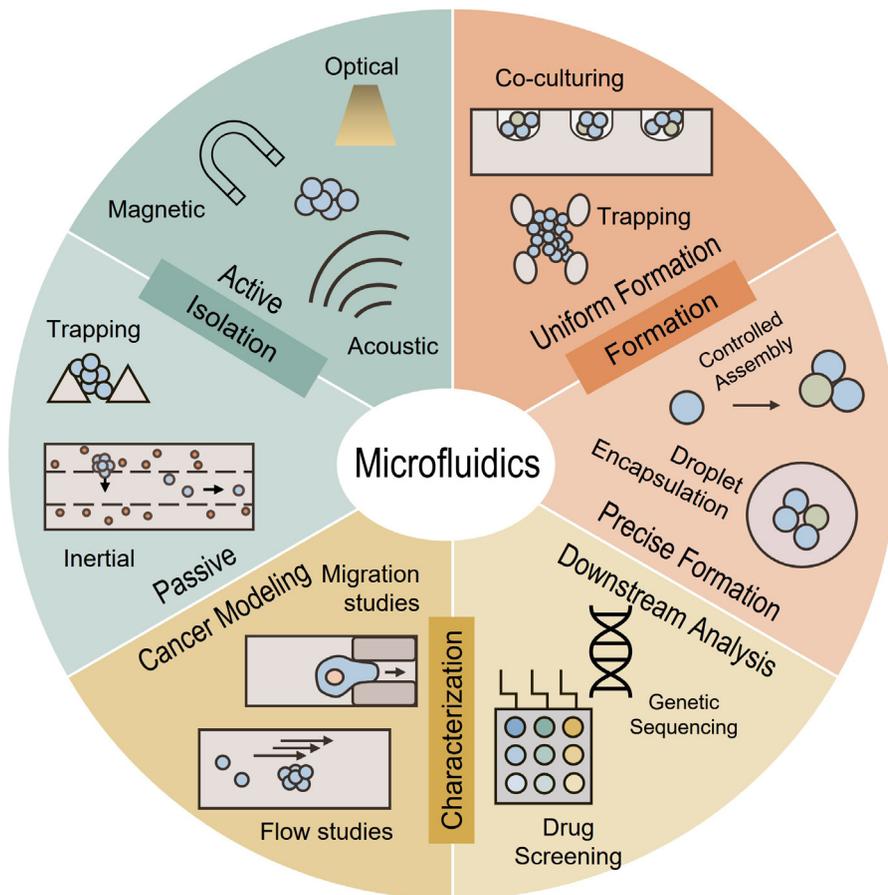


FIG. 1. Microfluidic isolation, formation, and characterization of CTC clusters for cellular and molecular investigations of cancer metastasis or disease monitoring.

from either expanded patient-derived cells or cancer cell lines integrated with downstream processes such as drug screening, molecular profiling, and/or cancer modeling. Differences between primary and cell line-formed clusters may also need more investigation; therefore, conducting both methods may be done in complementary with each other for more physiologically accurate modeling of CTC clusters. A combination of *in vitro* metastasis assays, *in vivo* preclinical models, and analysis of patient-derived cells could potentially elucidate the process of metastasis and lead to more effective cancer therapies.

In this review, we will highlight the microfluidic techniques in isolating, forming, and characterizing CTC clusters from patient-derived samples, preclinical *in vivo* models, and cancer cell lines. Others have already covered the microfluidic strategies for *in vitro* organoids and cell spheroid formations for tumor modeling or drug screening applications.^{12–14} However, these discussions revolve around modeling solid tumors and their microenvironment using miniature tissue constructs. While increased attention has been paid to the topic of CTC clusters, few reviews specifically focus on CTC clusters. Indeed, some reviews discuss cell isolation and enrichment techniques^{9,23,24} while others focus exclusively on drug screening in solid and liquid biopsies.²⁵ In this review, our goal is to highlight the contributions of microfluidics in all three aspects: isolation, formation, and characterization of CTC clusters. We will first provide a brief

discussion of some studies around the biology of CTC clusters and then illustrate the role microfluidics can play in elucidating metastatic mechanisms in the hope of developing improved therapies. Finally, we will discuss the current challenges and future perspectives of microfluidic technologies for cancer and metastasis research.

II. CTC CLUSTERS FOR CANCER RESEARCH

CTCs can offer much real time information on the status of cancer in a patient. These cells have been used in *in vivo* models of metastasis to further elucidate the process and aid in developing treatments. Studies have provided evidence that CTC clusters detach as multicellular aggregates from the primary tumor and hold necessary information on the pathways of metastasis.² It is, thus, of importance to identify the phenotypic traits and molecular features of CTCs and CTC clusters that facilitate metastasis seeding and the basis of the interactions of CTCs with other cell types such as immune and stromal cells.

In addition to CTC clusters, other cellular aggregates used for modeling of solid tumors include organoids and spheroids. Organoids are 3D self-organizing tissue constructs, usually embedded in a 3D matrix, derived from healthy and tumor tissue samples,²⁶ CTCs,²⁷ or engineered with embryonic or induced pluripotent stem cells.^{28,29} Organoids also offer flexibility, as they are compatible with high-throughput analyses and are amenable to genetic modification.²⁴

Similarly, spheroids are aggregates of cells with a uniform geometry that mimics small tissue lesions but are less complex than organoids.³⁰ There are fundamental differences in the structure and microenvironment between most cell spheroids and CTC clusters (Table I). Tumor spheroids exhibit steep gradients, cell-matrix attachments, and a necrotic core mimicking tumor areas away from blood vessels, which may not necessarily reflect the properties and behavior of CTC clusters.^{11,16} Therefore, careful design must be considered to better represent the characteristics of intravascular cell clusters that are exposed to other blood cells and fluidic stresses during their transit in circulation.³¹

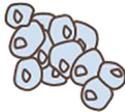
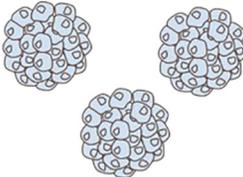
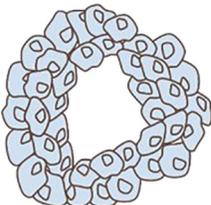
The main interest in studying CTC clusters and their importance includes the understanding of interactions between CTCs and blood cells,³² identifying the characteristics that CTCs attain during their transit in the bloodstream,³³ the prognostic value of CTC cluster analysis for personalized medicine,⁶ and the efficacy of anti-cancer drugs in targeting CTC clusters.²⁵ Although much has yet to be learned on the specific mechanisms of the metastatic process, some studies have slowly begun to reveal some insights. Preclinical mouse models have been vital in many of these mechanistic studies of metastasis;^{34–36} therefore, isolation of CTCs from them is routinely done in the research setting. Studies reveal that CTC clusters can exhibit partial epithelial-to-mesenchymal transition (EMT), which may be associated with the suppression of anoikis since they are able to retain their

intercellular junctions, and therefore, maintain physical stability. EMT switch of CTCs may also be modulated by survival and drug resistance pathways initiated by treatment.³⁷ The presence of other leukocytes in the bloodstream could even serve as a layer of protection during their transit.³² Contact between platelets and CTCs is sufficient to induce metastatic gene expression signature, an EMT-like transformation, and invasive behavior.³⁸

Tumors inherently exhibit significant genetic and transcriptomic heterogeneity as well as in epigenetics where inherent stochasticity of transcription may affect gene expression.^{39,40} In comparing the transcriptome profiles of neutrophil-associated CTCs against those of CTCs alone, *Szczerba et al.*³⁴ observed differentially expressed genes in CTC-neutrophil clusters that displayed enrichment in positive regulators of cell cycle and DNA replication compared to lone CTCs, which lead to the efficient formation of metastasis.³⁴ These findings motivate the need for even more mechanistic studies to fully uncover the metastatic process. Many *in vitro* studies of CTCs and metastasis have benefited from microfluidics through isolation, culture, and analysis systems.

Challenge arises in the preservation of viability for *ex vivo* CTC analysis. *In vivo* studies have shown that it only takes a few viable cells to survive circulation for metastasis to occur.^{41,42} In patient samples, CTC numbers and viability can vary as well (<10–1500 CTCs breast⁴³ and non-small cell lung cancer patients^{43,44}). Metastatic propensity

TABLE I. Comparison of cellular formations in cancer research.

	Cell aggregates			
	Single cells	Clusters	Spheroids	Organoids
				
Characteristics	Single cells may be isolated by physical-based or affinity methods Useful in single-cell resolution next-generation sequencing Amenable for high-throughput technologies in imaging, mass spectrometry and DNA- and RNA-sequencing	Aggregates of cancer cells that depart from solid tumors and enter the bloodstream. ⁸⁶ Isolated from whole blood or formed <i>in vitro</i> . CTC clusters display distinct gene expression profiles and dissemination modes compared to single CTCs and play a role in cancer progression. ⁸⁶	3D spherical cell aggregates that model <i>in vivo</i> solid tumors. Larger spheroids have central necrosis and regions of hypoxia. ¹² Can be formed <i>in vitro</i> by 3D culture, hanging drop, microwell, droplet Mimic tissues with cell–cell interactions as well as diffusional limits to mass transport of drugs, nutrients, and other factors. ¹²	3D self-organizing cellular constructs with tissue structure and function. Derived from adult organs or embryonic and adult stem cells. ¹³⁴ Demonstrate functionality such as muscle contractility, epithelial barrier function, neuronal activity, hepatocyte detoxification, gastric acid secretion, and secretion of insulin by beta cells. ¹³⁴
Related microfluidic reviews	Pei <i>et al.</i> ¹¹ and Sharma <i>et al.</i> ¹³⁵	Sharma <i>et al.</i> ¹³⁵	Mehta <i>et al.</i> , ¹² Moshkayan <i>et al.</i> , ¹³ and Vadivelu <i>et al.</i> ¹⁴	Ayuso <i>et al.</i> , ¹³⁶ Duzagac <i>et al.</i> , ¹³⁷ Kim <i>et al.</i> , ¹³⁸ and Velasco <i>et al.</i> ¹³⁹

can be influenced by the physical characteristics that single CTCs and CTC clusters possess, and metastasis may favor the survival and growth of a few subpopulations of these cells.⁴¹ Cancer cells may also exploit and/or be resistant to the physical forces present in the circulation to successfully seed in distant metastases.^{45,46} For example, flow rates, vessel size, circulating time, and shear stress can influence the survival of CTCs and control seeding patterns.^{47,48} These factors, therefore, might be of importance when dealing with CTCs *in vitro* and/or *ex vivo*. In fact, some have started to study these influences using microfluidic methods to induce such physical parameters.^{19,45,48,49}

Another important aspect to consider is reliable methods of assessment of viability. In *ex vivo* capture, only a small fraction of captured CTCs will be viable for cell analysis and cell expansion.⁴¹ This necessitates for high rates of cell viability in microfluidic devices. In *ex vivo* studies, the cell release and transfer of samples and reagents can contribute to the death of a few cells. Indeed, for microfluidic capture systems, previous works have shown over 97% cell viability is achieved in microfluidic platforms.⁵⁰ In triangular traps, the release of clusters

under 250 ml/h reverse flow had no negative effect on cell viability.⁵¹ In another example, LM2 cells had achieved up to >97% viability in a microwell valve system.²¹

Microfluidic systems have also made evaluation of CTCs and CTC clusters achievable in a high-throughput manner with the ability to recapitulate the cancer microenvironment (Fig. 2). Ideally, the integration of CTC analyses with isolation and cell culture could make these microfluidic systems functional in the clinical setting. Below, we summarize the microfluidic techniques used in obtaining and analyzing CTC clusters for cancer research.

III. ISOLATION OF CTC CLUSTERS WITH MICROFLUIDIC SYSTEMS

Microfluidic isolation technologies make it possible to extract primary cell specimens from patient blood that can be used for biological investigations and disease diagnosis and/or prognosis. In addition, these systems may be integrated with cell culture systems to expand patient-derived cells and develop CTC cell lines for prolonged *ex vivo* studies such as cell invasiveness and metastatic competency.⁵² The two

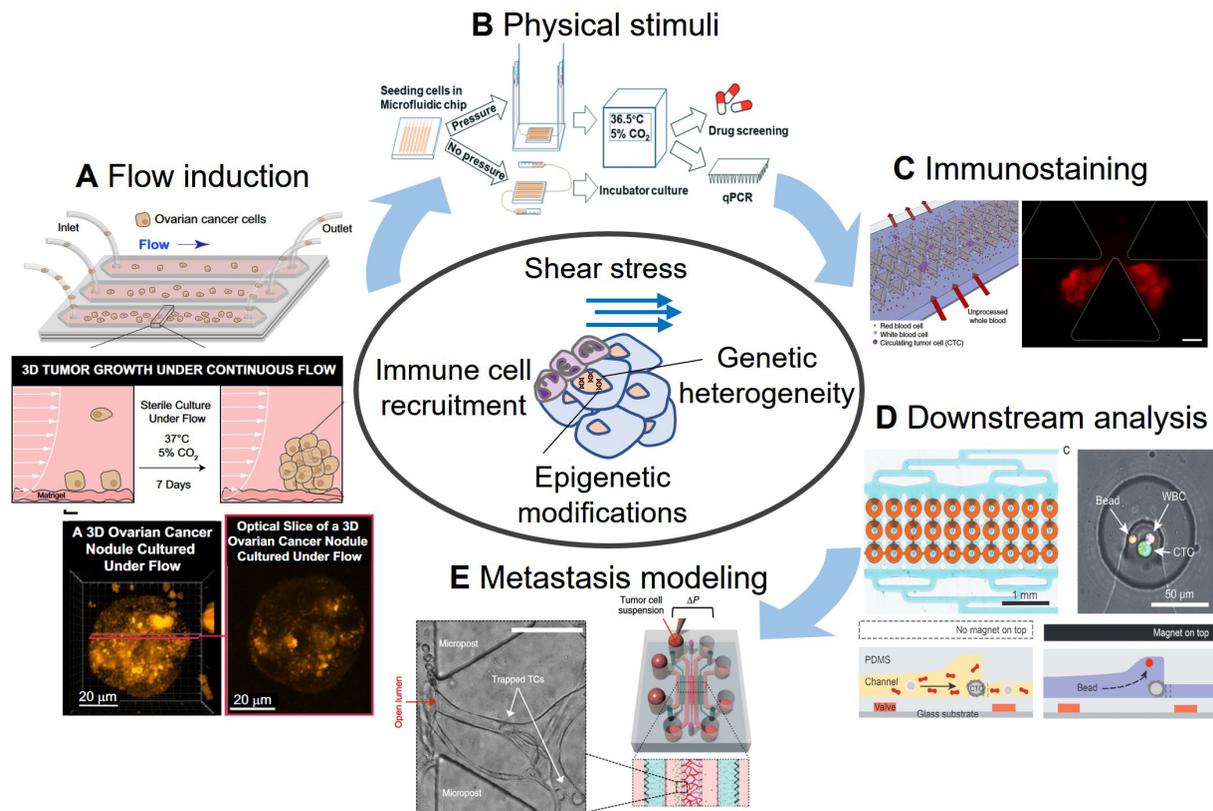


FIG. 2. Microfluidic techniques to evaluate relevant factors affecting CTC clusters. (a) Flow experiments in microfluidic chip to study the effect of sustained flow on the growth and molecular features of 3D ovarian cancer nodules. Reproduced with permission from Rizvi *et al.*, Proc. Natl. Acad. Sci. **110**, 22 (2013). Copyright 2013 Authors, licensed under a Creative Commons Attribution (CC BY) license.¹⁹ (b) Study of hydrostatic pressure effects on human breast cancer cell drug resistance. Republished with permission from Shang *et al.*, Lab Chip **21**, 4 (2021). Copyright 2021 Royal Society of Chemistry and Copyright Clearance Center, Inc.¹¹¹ (c) On-chip immunostaining of CTC clusters after capture. Reprinted by Sarioglu *et al.*, Nat. Methods **12**, 7 (2015). Copyright 2015 Springer Nature Customer Service Center GmbH.⁵¹ (d) Microfluidic protein quantification using a bead-based immunoassays. Reproduced with permission from Armbricht *et al.*, Adv. Sci. **7**, 11 (2020). Copyright 2020 Authors licensed under a Creative Commons Attribution (CC BY) license.²¹ (e) *In vitro* model of microcirculation to study cell arrest, transendothelial migration and early micrometastases formation. Reprinted with permission from Chen *et al.*, Nat. Protoc. **12**, 5 (2017). Copyright 2017 Springer Nature Customer Service Center GmbH.¹⁴⁰

main methods used to obtain primary cells are the solid and liquid biopsy. Solid biopsy is the traditional sampling method, where a small amount of tissue is extracted from the body to obtain patient-derived primary cells. Post-analysis of these tissues requires their dissociation into single cells, which may be done chemically, mechanically, or through microfluidics.⁵³ However, solid biopsy is an invasive technique that cannot be repeated frequently and can present challenges depending on the anatomical structure of the tumor or its location. Therefore, much attention has been paid to liquid biopsy, which involves extraction of bodily fluids, such as blood or pleural effusion, for analysis of biomarkers, including CTCs, circulating tumor DNA (ctDNA), and extracellular vesicles (EVs).⁵⁴

Liquid biopsy is gaining popularity due to its minimally invasive nature, which allows for routine disease monitoring over time and more frequently. Microfluidic techniques are especially advantageous in liquid biopsy as they only require small sample volumes and minimal manual pipetting. In addition, they can be integrated for further downstream molecular analysis, *in vitro* studies, or tracking of tumor response to treatment. Microfluidic methods of isolating CTCs and CTC clusters include sized-based,^{55,56} marker-dependent,^{18,57} and active techniques.⁶⁰ These are summarized in Table II.

Some techniques involve integration of cell sorting with trapping,⁵⁰ where after sorting, cells or clusters may be trapped using microwell, micropost, microfiltration, or microchamber methods without external force.⁶¹ Isolation solely by trapping is also an effective method of CTC capture.⁶¹ For example, Hosokawa *et al.*⁶² used a microcavity array system to capture single and clustered CTCs.⁶² Sarioglu *et al.*⁵¹ developed the cluster chip, a micropillar device made of bifurcating traps specifically to trap cell clusters from unprocessed blood.⁵¹ These capture methods immobilize single cells and clusters in a controllable and independent manner enabling single-cell analysis.⁶¹ Microfiltration has also been used in CTC capture, which creates less mechanical stress on the CTCs.^{62–64} Zhou *et al.*⁶⁴ achieved a high

capture efficiency of 78%–83% and a cell viability of 71%–74% in their separable bilayer microfiltration device.

Other isolation methods are sized-based techniques^{55,56} that avoid complications related to biomarker inconsistency since detection is not based on specific surface markers but rather use other physical properties such as size or deformability to isolate target cells.⁶⁵ One label-free method is the deterministic lateral displacement (DLD),⁶⁶ a passive technique that separates particles based on size. Au *et al.*⁴ used the DLD method in a two-stage device with the first stage separating large clusters and the second stage for smaller clusters that were initially deflected by the first stage using asymmetric pillars [Fig. 3(a)].⁴ This technique preserved the integrity of clusters while also minimizing damage that may lead to processing bias. Edd *et al.*⁶⁷ developed a non-equilibrium inertial separation array that combines inertial focusing with repetitive flow-shifting isolating CTC clusters from large volumes of minimally diluted whole blood [Fig. 3(b)].⁶⁷ The device isolated spiked CTC clusters from >30 ml/h of whole blood with 80% efficiency and an on-chip yield of ~100%.⁶⁷ However, the main disadvantage of the size-based separation methods is the loss of smaller cells, which can result in loss of valuable information from the patient.⁶⁸

Centrifugal forces with Dean flow also enable the continuous focusing of larger cells.^{55,56} Hou *et al.*⁶⁹ used a spiral channel isolation device exploiting Dean migration and inertial separation in curvilinear channels, achieving >85% separation efficiency [Fig. 3(c)]. Capture of intact clusters of MCF-7 breast cancer cells was achieved even with high flow conditions due to the short transit time within the channel, which prevented the breakup of clusters.⁶⁹ In another method developed by the Di Carlo group,⁷⁰ microscale vortices and inertial focusing were employed to passively isolate and concentrate larger cells [Fig. 3(d)].⁷¹ They optimized this method for high-purity extraction by systematically varied parameters, including channel dimensions and flow rates, to arrive at an optimal device for maximum trapping efficiency

TABLE II. Methods for microfluidic isolation of CTCs and clusters.

	Principle	Efficiency	Purity	Throughput	Advantages	Disadvantages
Sized-based trapping	Hydrodynamic capture through microwells ^{92,93} and micropost trapping ⁵¹	~40–99% (Increased efficiency with increasing cluster size)	...	200 ml/min, –2.5 ml/h	High-efficiency, label-free	Low-throughput, low purity, and low specificity
Sized-based flow separation	Deterministic lateral displacement (DLD), ⁴ shear-induced diffusion (SID), ^{50,72} and inertial focusing ^{3,67–69,73}	~37%–99%	57%–94%	0.2–0.5 ml/min	High-throughput, high-efficiency, label-free	Low specificity, low-mid purity
Marker-dependent	Antibody ^{1,58,75,76,141} and nanoparticle ⁵⁹ coated surfaces	~60%–98%	~14%–86%	1–2.5 ml/h	High specificity	Low throughput, low purity, low efficiency, difficult to retrieve cells
Active	Capture through optical, ⁸³ magnetic, ⁸⁴ acoustic, ^{78,79,82} and dielectrophoretic ^{83,85}	~71%–90%	84%–91.5%	1.2–30 ml/h	High purity, minimized shear force, label-free	High efficiency, low throughput, complex setup

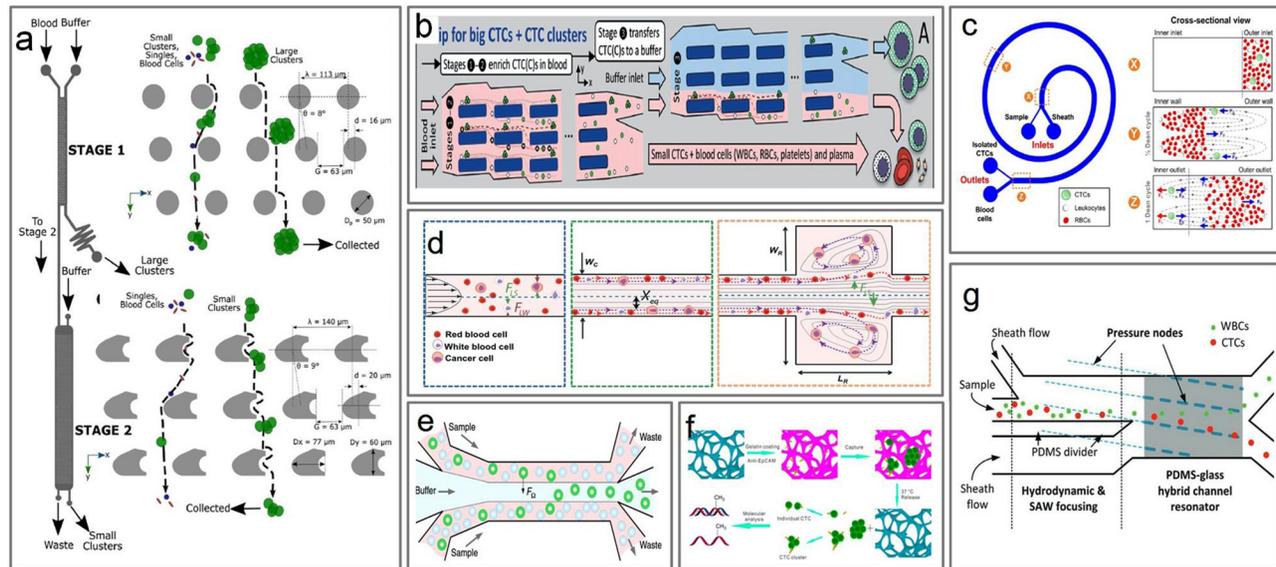


FIG. 3. Microfluidic capture of single and clustered CTCs. (a) Two-stage cluster capture array of cylindrical micropillars. Stage 1 deflects large clusters while stage 2 deflects small clusters from other blood cells using deterministic lateral displacement. Reproduced with permission from Au *et al.*, *Sci. Rep.* **7**, 1 (2017). Copyright 2017 Authors, licensed under a Creative Commons Attribution (CC BY) license.⁶³ (b) Size-based CTC and cluster sorter built from enlarged non-equilibrium inertial separation arrays. Republished with permission from Edd *et al.*, *Lab Chip* **20**, 3 (2020). Copyright 2020 Royal Society of Chemistry and Clearance Center, Inc.⁶⁷ (c) CTC isolation using the spiral inertial microfluidic device where smaller cells migrate toward the inner wall, then back to outer wall again, while the larger CTCs experience additional strong inertial lift forces and focus along the microchannel inner wall. Reproduced with permission from Hou *et al.*, *Sci. Rep.* **3**, 1 (2013). Copyright 2013 Authors, licensed under a Creative Commons Attribution (CC BY) license.⁶⁹ (d) In the Vortex chip, particles migrate to lateral equilibrium positions depending on the channel cross section, where the wall effect is reduced in the reservoir. Republished with permission from Sollier *et al.*, *Lab Chip* **14**, 1 (2014). Copyright 2014 Royal Society of Chemistry Clearance Center, Inc.⁷¹ (e) Inertial separation of CTCs and clusters, where they are focused to the center of a straight channel. Reproduced with permission from Zhou *et al.*, *Microsyst. Nanoeng.* **5**, 1 (2019). Copyright 2019 Authors, licensed under a Creative Commons Attribution (CC BY) license.⁷³ (f) Capture and release of CTC cluster in a 3D scaffold chip. Reproduced with permission from Cheng *et al.*, *Anal. Chem.* **89**, 7924–7932 (2017).⁷⁶ Copyright 2017 American Chemical Society. (g) Acoustic separation of CTCs from WBCs. The cells are separated due to difference in the lateral shift. Republished with permission from Wu *et al.*, *Small* **14**, 32 (2018). Copyright 2018 John Wiley and Sons, Clearance Center, Inc.⁸²

and purity (57%–94% purity).⁷¹ Kulasinghe *et al.*³ employed inertial migration using a simple straight channel device to isolate head and neck CTCs' clusters from whole blood followed by DNA fluorescence *in situ* hybridization (FISH) [Fig. 3(e)].³ As another method of passive separation, Hayashi *et al.*⁶³ used shear-induced diffusion (SID) of cells from concentrated suspensions.⁶² This technique was used to separate CTCs from unprocessed whole blood in a straight, rectangular microfluidic channel integrated with a cell trapping and culture chamber. The device integration effectively streamlines cell separation, capture, staining, or *in situ* culture with little manual interference.⁶³

Marker-dependent techniques for cell isolation¹⁸ make use of epithelial cell adhesion molecule (EpCAM) expressed on the surface of tumor cells.^{1,74} This technique is also applied in the first CTC isolation system, CellSearch, to be approved by the U.S. Food and Drug Administration (FDA). Anti-EpCAM magnetic particles conjugate to tumor cells and be immobilized by a magnetic force.¹ In microfluidic devices, surface functionalization with antibodies allows for marker-dependent capture of CTCs and CTC clusters. Stott *et al.*⁵⁸ developed the herringbone (HB)-chip with surface-lined anti-EpCAM antibodies that captured CTCs and CTC clusters with $79\% \pm 4.5\%$ efficiency at 0.12 ml/h flow rate.⁵⁸ The herringbone design induces microvortices that disrupt the laminar flow streamlines and, therefore, increase the number of cell interactions with the antibody-coated surfaces. Jiang

*et al.*⁷⁵ then used the HB-chip in a two-step process for platelet covered CTC-leukocyte cluster isolation based on immunoaffinity to platelets.⁷⁵ Microfluidic devices can also be scaffolded for a 3D capture of CTC clusters.^{57,76} For example, Cheng *et al.*⁷⁶ achieved >80% capture efficiency and 60%–70% recovery ratio in spiked samples, followed by DNA and RNA methylation analysis of the cells [Fig. 3(f)]. To perform downstream analysis right after capture, it is then necessary to release cells from the surface. A ligand-exchange reaction was used by Park *et al.*⁵⁹ using a modified the HB-chip with gold nanoparticle coating to allow for easy detachment of the captured CTCs. The metal–thiol interactions can be disrupted in the presence of excess thiol molecules that resulted in the release of cells.⁵⁹ Though these marker-dependent techniques can be highly specific, a limitation to this approach is the reduced efficiency due to downregulation EpCAM in some CTCs. Therefore, alternative methods that do not require the EpCAM expression may be necessary to isolate some CTCs and CTC clusters.⁷⁷

So far, we have focused on passive separation methods, yet active methods can too be used in a label-free format and can maintain the integrity of cells and clusters.^{78,79} Active methods⁶⁰ use external force such as acoustic, magnetic, optical, or dielectrophoretic to separate and isolate cell clusters from blood or buffer. The acoustic-based method uses standing surface acoustic waves to gently separate CTCs from white blood cells (WBCs), preserving the phenotype and

genotype of the cell.^{80,81} Wu *et al.*⁸² successfully applied this technique to separate CTCs and CTC clusters from blood samples collected from patients with metastatic prostate cancer.⁸² Chiu *et al.*⁸³ used optically induced dielectrophoresis (ODEP) to separate CTC clusters [Fig. 3(g)].⁸³ Their device used alternating current voltage between the top and bottom surfaces of the ODEP system and generated a non-uniform electric field when illuminated. The electric field interacts and manipulates an electrically polarized microparticle where >80% purity is achieved.⁸³ Lin *et al.*⁸⁴ applied lateral and vertical magnetic force to separate white blood cells from whole blood at a flow rate of $20 \mu\text{l min}^{-1}$ and achieved the final separation purity $93\% \pm 1.7\%$ and viability up to $97.5\% \pm 1.8\%$. Do *et al.*⁸⁵ developed a microfluidic DEP using a gold electrode structure, which is used to concentrate cells suspended in the chamber by DEP and a stepping electric field. The target cells are captured by an electrode immobilized by anti-EGFR, which has high affinity toward the target cells. In general, active methods are usually high in efficiency; however, compared to passive methods, some major drawbacks to these systems are that they are more complex in operation, usually lower in throughput, and require pretreatment of blood.

Microfluidic isolation systems have progressed in recent years and have demonstrated efficient separation of target cells. In addition, many improvements have been made in applying these systems to the isolation of extremely rare cells from biological samples, proving their value in the clinical and laboratory setting. As discussed here, there are advantages and disadvantages to each method of isolation, and therefore, careful decision must be made for specific applications. However, even with advancements in these methods, due to the rarity of CTC clusters, the sole isolation of these cells is not sufficient for extensive research and/or high-throughput screening. In Sec. IV, we discuss potential ways to form and expand such cell clusters to extend their use in the laboratory.

IV. FORMATION OF CTC CLUSTERS WITH MICROFLUIDICS

The potential clinical significance of CTC clusters motivates the need for accurate *in vitro* cancer models to survey how CTC clusters survive and how to target them effectively with anti-metastatic treatments. In general, CTC clusters can either be homotypic or heterotypic, where heterotypic clusters can include other immune and stromal cells. Heterotypic clusters are much rarer compared to single CTCs and homotypic CTC clusters.⁸⁶ Although rare, studies suggest that heterotypic clusters may be significant in initiating metastasis.^{34,86} Therefore, it is imperative to explore the potential of these clusters as clinical tools for research. Simple microfluidic systems have been proven effective in the culture of single cells⁸⁷ and *in vitro* formation of cell spheroids.⁸⁸ Other methods of aggregate formations have been performed using hanging drop methods,⁸⁸ liquid marbles,⁸⁹ non-adherent plates,^{30,51} and droplet formation⁹⁰ techniques; however, microfluidic techniques offer much more uniform and precise formation of CTC clusters.

Forming *in vitro* CTC clusters with readily available cancer cell lines can also be used for both investigation of cell cluster behavior and drug screening where cell response can be assessed in a very high-throughput manner. For example, cancer cell lines, such as NCI-H187 (small cell lung cancer) and NCI-H2122 (non-small cell lung cancer), exhibit similar features as primary CTCs and, therefore, can be

alternatives for patient-derived CTCs.³¹ Although these may not completely replace primary cells, they can be used for optimizing *in vitro* models as well as complementing preclinical mouse studies and clinical sample analysis. Moreover, microfluidic cell sorting and capture devices can conveniently be integrated with on-chip cell culture systems⁵⁰, to ease the processing of samples. Perhaps the simplest approach in forming clusters would be the use of microwells, which relies on the sedimentation of cells into individual compartments allowing for fine spatial control. Spheroid and organoid formations are commonly formed with microwell techniques.^{15,16,22} Jung *et al.*¹⁵ established lung cancer cell organoids derived from small-cell lung cancer (SCLC) patients in a microwell system that is able to load, expand, and identify drug responses under physiologically relevant flow conditions.¹⁵ Dadgar *et al.*^{16,22} demonstrated the formation of spheroids with a relatively low seeding density, demonstrating the utility of the microfluidics to be used with the limited cell numbers. Microwells also allow for high-throughput single-cell analysis due to the ease in the fabrication of many individual compartments in one chip. Size and cross-sectional profiles of microwells can be easily tuned to meet the requirements for specific cell culture applications such as the formation of CTC clusters.²²

Parameters that contribute to the proper formation of cell clusters in microwells include cell seeding density, microwell dimensions, and device surface properties. Tu *et al.*⁹¹ used CO₂ laser ablation on polystyrene substrates to create concave microwells for the formation of A549 lung cancer cell clusters that are about $50 \mu\text{m}$ in size [Fig. 4(a)]. In their study, A549 cell aggregates were successfully generated in polystyrene microwells, where aggregate size was controllable and able to form about $\sim 40\text{--}80 \mu\text{m}$ -sized aggregates.⁹¹ It has also been demonstrated to form cell clusters directly after extraction from patient blood through red blood cell (RBC) lysis. Short term culture of clusters after RBC lysis from multiple cancer types was achieved by Balakrishnan *et al.*⁹² using ellipsoidal agar microwells with cultures maintained for 3 weeks. They assessed the expressions of cytokeratins (CK) in clusters that vary in compactness and found that tighter clusters showed greater therapy resistance.⁹² Khoo *et al.*⁹³ created a microwell system capable of co-culturing CTCs with the white blood cells from the same patient.⁹³ The device consisted of three layers, including a concentration gradient generator, a barrier layer that separates each column of inverted dome-shaped elliptical microwells into an individual channel, and a layer of microwells that contain the CTC clusters. This technology can derive CTC clusters from liquid biopsies without prior enrichment of the CTCs.⁹³

For the precise formation of heterotypic clusters, more complex systems involving valving, trapping, and/or droplet formation techniques are used. Microvalves can facilitate controlled cell culture and drug assays such as the work of Desyatnik *et al.*,⁹⁴ where pneumatic microvalves were combined with cell culturing of MCF-7 and 293T cells and drug microarray [Fig. 4(b)]. The cells were allowed to aggregate and then cultured for 24 h where they found substantial cell clustering.⁹⁴ Fatsis-Kavalopoulos *et al.*⁹⁵ created a hydrodynamic trapping system to form four-cell clusters consisting of one pancreatic β -cell and three breast cancer cells. Single-cell traps were typically occupied within seconds of the first cells entering the chip, while loose cells were recovered at the cell outlet.⁹⁵ This technique can especially be useful for modeling microtissue niches and enables tailored cell assemblies. Because of the difficulty of obtaining and maintaining

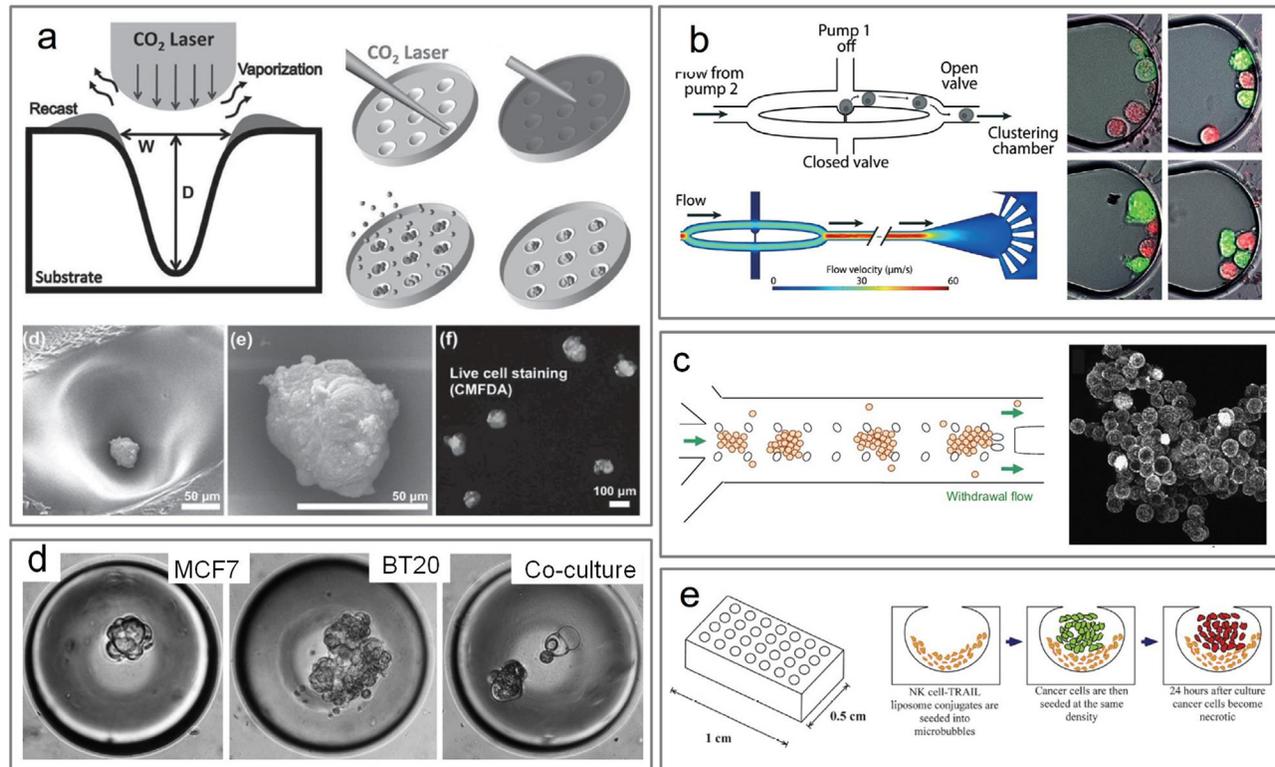


FIG. 4. *In vitro* formation of clusters in microfluidic devices. (a) Laser ablation fabrication process of concave microwells for the formation of 3D aggregates. Republished with permission from Tu *et al.*, *Adv. Healthcare Mater.* **3**, 4 (2013). Copyright 2013 John Wiley and Sons, Clearance Center, Inc.⁹¹ (b) Ejection of cells from single-cell traps where lock arrows denote the flow paths of cells dislodged from the trap flowing toward the clustering chamber. Republished with permission from Fatsis-Kavalopoulos *et al.*, *Lab Chip* **19**, 6 (2019). Copyright Royal Society of Chemistry Clearance Center, Inc.⁹⁵ (c) Schematic illustration of *in situ* formation and immobilization of 3D multi-cellular aggregates in a microchannel. Reprinted with permission from Ong *et al.*, *Biomaterials* **29**, 3237–3244 (2008). Copyright 2008 Elsevier.¹⁰⁰ (d) Bright field images of CTC clusters grown in PDMS microbubbles. Reproduced with permission from King *et al.*, *Am. J. Physiol.-Cell Physiol.* **308**, 10 (2015). Copyright 2015 Authors, licensed under a Creative Commons Attribution (CC BY) license.¹⁰¹ (e) Cell culture microbubbles formed in PDMS for lymph node micrometastases model. Republished with permission from Chandrasekaran *et al.*, *Lab Chip* **14**, 1 (2014). Copyright 2014 Royal Society of Chemistry Clearance Center, Inc.¹⁰²

CTC–neutrophil clusters *ex vivo*, the formation of CTC–neutrophil clusters *in vitro* could be a significant step for understanding the metastatic mechanisms of heterotypic clusters. Park *et al.*⁹⁶ employed an inertial-force-assisted droplet microfluidic chip with double spiral channels to recapitulate CTC–neutrophil clusters. The deterministic encapsulation of cells facilitated the pairing of neutrophils and cancer cells with varying ratios. The encapsulated cells spontaneously formed clusters and showed well-defined molecular signatures of CTC–neutrophil clusters.⁹⁶

Antibody-coated devices are also used for the formation of clusters.^{97–99} For example, Chen *et al.*⁹⁷ created a membrane mimetic microfluidic device with antibody-conjugated supported lipid bilayer smart coating to capture viable CTCs and clusters directly from whole blood and demonstrated *ex vivo* culture over weeks. The device was able to promote dynamic clustering of lipid-tethered antibodies to CTC antigens and minimize nonspecific blood cells retention. In another study, capture and culture of PC3 prostate cancer cells were presented by Bichsel *et al.*,⁹⁸ where they expanded clonal PC3 cells on

functionalized microwells. They then injected hydrogel matrix formulation into the wells. The hydrogel was degradable by matrix metalloproteinases (MMPs), which is important for 3D cell invasion during metastasis. The hydrogel was used to mimic the extracellular matrix (ECM) surrounding the tumor and to facilitate cell proliferation. The cells were then assessed by microscopy over one week in culture.⁹⁸

Microfluidic formation of cell clusters is also feasible without requiring the functionalization or ECM coating of surface areas. Ong *et al.*¹⁰⁰ developed a gel-free microfluidic trapping method for 3D culturing of A549 and C3A cell lines and primary bone marrow mesenchymal stem cells [Fig. 4(c)]. This simple device was successful in the formation and immobilization of cell aggregates lodged between microposts in a straight channel.¹⁰⁰ King *et al.*¹⁰¹ created microstructures and cultured BT20, MCF-7, and MCF10A aggregates, mimicking tumor heterogeneity by combining all cell lines in a 1:1:1 ratio. The cell cultures yielded a seeding density of 1–15 cells per microstructure in which the seeding density was dependent on incubation time during seeding [Fig. 4(d)]. They also achieved one to five cell clusters per

microstructure demonstrating physical similarity of clinically obtained CTCs and CTC aggregates.¹⁰¹

The co-culturing approach has also been done to investigate lymph node micrometastasis with cancer cells and natural killer (NK) cells.¹⁰² Chandrasekaran *et al.*¹⁰² created a cell culture device to investigate the efficacy of NK-mediated therapy for targeting lymph node micrometastasis. They fabricated spherical cavities using deep reactive ion etched (DRIE) silicon wafers to form the structures in polydimethylsiloxane (PDMS) by gas expansion molding [Fig. 4(e)].¹⁰² This technique generated rounded microbubble structures, mimicking deep cortical units of a lymph node and creating clusters about 80–100 μm in diameter.¹⁰² Choi *et al.*¹⁰³ described an early-stage breast cancer chip model that enabled co-culture of breast tumor spheroids with human mammary ductal epithelial cells and mammary fibroblasts in a compartmentalized 3D structure. This device can be used to evaluate the efficacy and toxicity of an anticancer drug.¹⁰³ Hsiao *et al.*¹⁰⁴ used a two-layer microfluidic device to co-culture prostate cancer cells, osteoblasts, and endothelial cells from 3D cancer tumors. This method ensures uniform incorporation of all co-culture cell types into each spheroid and keeps the spheroids stationary for easy tracking.¹⁰⁴

These advancements in microfluidic systems can provide larger quantities of rare cells and clusters *in vitro* and, therefore, offer great promise in expanding cancer metastasis studies. Because formation of such clusters with microfluidics allows controllable cell grouping, it is then possible to create heterotypic CTC clusters, making microfluidics more advantageous among other conventional methods. Successful *ex vivo* expansion of CTC clusters will also enable drug screening and personalized medicine applications. The main challenge now for microfluidic systems is to maintain cell viability, which is usually of concern with patient-derived CTCs.

V. MICROFLUIDIC SYSTEMS FOR CHARACTERIZATION AND ANALYSIS OF CTCs

Molecular analyses of CTC clusters can provide a deeper understanding of the metastatic process and are, therefore, conducted after isolation or formation of clusters. These molecular techniques include immunostaining, FISH, and real time quantitative polymerase chain reaction (RT-qPCR) (Table III). Single-cell analysis, such as next-generation sequencing (NGS) and mass cytometry technologies, may be done to characterize the genome, transcriptome, methylome, and proteome of tumor cells. RNA sequencing analysis was demonstrated on the CTC clusters captured with the Cluster Chip developed by the Toner group,⁵¹ suggesting some heterogenous characteristics of CTC clusters and association with other immune cells.⁵¹ Donato *et al.*³⁵ evaluated proteins that mediate hypoxia-driven clustering in *in vivo* mouse models. Single-cell technologies have uncovered insight to the genetic makeup of CTCs over the past few years, but limited cell numbers limit molecular analysis, especially if patient stratification is required.⁸⁶ In addition, challenges in single-cell sequencing include strong stochastic variation and high error rates derived from DNA amplification due to limited quantities of DNAs and mRNAs extracted from CTCs.⁸⁶ Nevertheless, some groups have already started to integrate the use of microfluidics in the downstream analysis of CTCs and CTC clusters. In this section, we describe existing technologies for the analysis of CTCs and CTC clusters.

Mouse models of metastasis offer a great platform for CTC and clusters research and have been combined with microfluidic

TABLE III. Parameters for characterization of CTCs and CTC clusters in microfluidics.

Microfluidic methods of CTC cluster analysis	Relevant parameters
Cell–cell adhesion assays ⁹⁸	Cell–cell adhesion forces
Co-culture ^{101–103,125,127,142}	Cell–cell interactions
Shear flow experiments ^{19,101,115}	Velocity lateral displacement within channels
High-resolution imaging ^{19,95,124} and immunofluorescence ^{19,74,92}	Cell viability, size and morphology, extravasation rate, protein expression, and molecular characteristics
RNA isolation and protein isolation, ¹⁹ qRT-PCR, ¹¹⁰ DNA sequencing, ¹⁰⁹ mass spectrometry, ³⁵ fluorescence <i>in situ</i> hybridization (FISH), ^{3,93} and surface protein expression ¹⁴³	Gene expression, protein expression, molecular characteristics, and proteomic profile

techniques for further CTC characterization. Williams *et al.*¹⁰⁵ developed diffuse *in vivo* flow cytometry (DiFC), an optical instrument that allows continuous, noninvasive counting green fluorescent protein expressing CTCs in large blood vessels in mice. They used DiFC to study short-term changes in CTC numbers in multiple myeloma and Lewis lung carcinoma xenograft models.¹⁰⁵ In an integrated microfluidic device capable of capture and isolation, Armbricht *et al.*²¹ conducted protein quantification secreted by CTCs. The device captures functionalized magnetic beads and single CTCs and CTC-WBC clusters in individual chambers for the assessment of protein secretion.²¹ A simple, label-free acoustofluidic device was developed by Bai *et al.*³⁶ from whole blood samples of mice implanted with 4T1 cells derived from BALB/c mice that expressed the firefly luciferase gene.³⁶ This study aimed to create a postoperative evaluation system based on the long-term dynamic detection of CTCs to help in guiding treatment in metastatic cancers.³⁶

In drug screening, throughput is greatly enhanced in microfluidics owing to the capacity for large numbers of compartments and parallelization. Balakrishnan *et al.*⁹² tested longitudinal treatment response of CTC clusters from patient samples of breast and lung cancer in microwells molded in agar [Fig. 5(a)]. They derived CTCs from cancer patients expanded without prior enrichment and maintained the culture under hypoxic conditions. Drug screening of dissociated solid tissue biopsies has been demonstrated by Eduati *et al.*,¹⁰⁶ where they used Braille valves to perform combinatorial drug screening. For organoid screening, Au *et al.*¹⁷ applied a microfluidic platform for culturing hepatic organoids generating arrays of individual, free-floating, 3D hydrogel-based microtissues.¹⁷ Tu *et al.*⁹¹ examined migratory behavior of A549 cell aggregates after a screen of drugs in 2D and 3D conditions showing differences in migration patterns between different dimensionalities. In addition, in studying cytotoxic drug resistance, A549 cell aggregates showed a 10–100-fold change in resistance compared to that of a monoculture.⁹¹ These results suggest that it is important to consider assay types and how they may affect the results of drug screening applications. Ability for increased microenvironmental

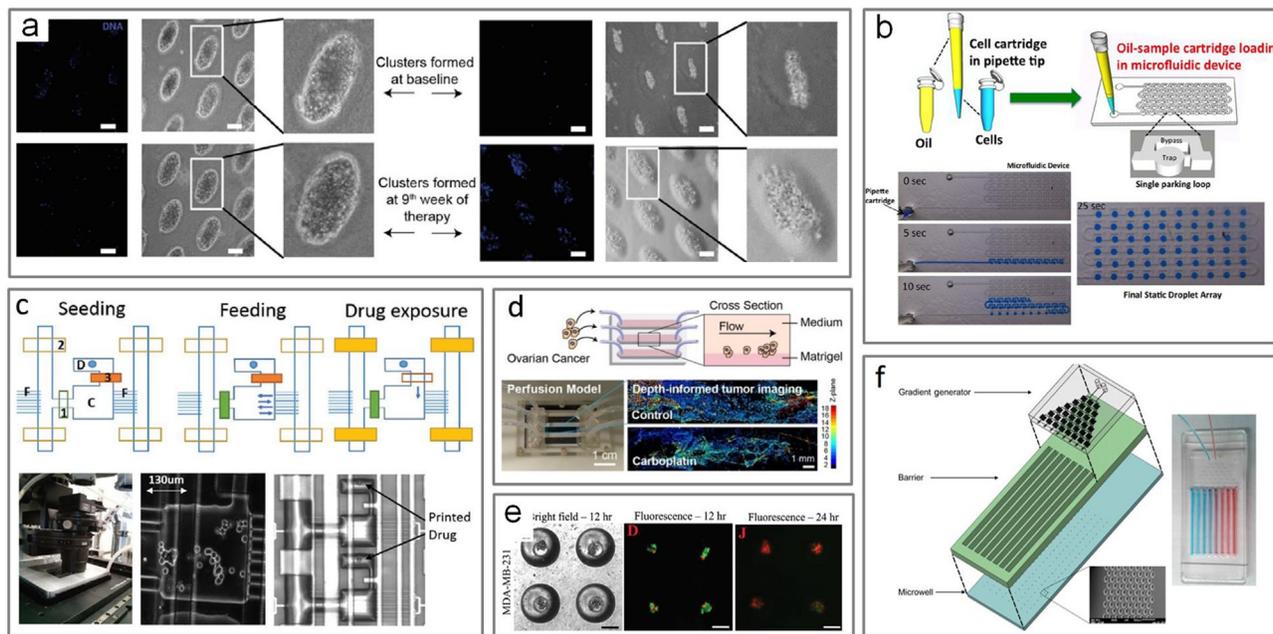


FIG. 5. Drug screening and evaluation of targeted therapies in microfluidic devices. (a) Cluster formation in cancer blood samples at baseline and after therapy in a lung cancer patient (left) and breast cancer patient (right). Reproduced with permission from Balakrishnan *et al.*, *Sci. Rep.* **9**, 1 (2019). Copyright 2019 Authors, licensed under a Creative Commons Attribution (CC BY) license.⁹² (b) Microfluidic drug resistivity assay showing preparation of cartridge by sequential aspiration of oil and cell-laden sample, followed by a single step dispensing into the microfluidic device. Reproduced with permission from Bithi and Vanapalli, *Sci. Rep.* **7**, 1 (2017). Copyright 2017 Authors, licensed under a Creative Commons Attribution (CC BY) license.¹⁰⁷ (c) Cell-culture microfluidic device and the drug chambers with printed drug inside. Republished with permission from Desyatnik *et al.*, *Adv. Biosyst.* **3**, 11 (2019). Copyright John Wiley and Sons Clearance Center, Inc.⁹⁴ (d) Schematic of a perfusion model used to study the impact of fluid flow on treatment resistance and molecular features of 3D ovarian cancer nodules. Reproduced with permission from Khoo *et al.*, *Br. J. Cancer* **120**, 407 (2019). Copyright 2019 Authors, licensed under a Creative Commons Attribution (CC BY) license.¹¹⁷ (e) Cells seeded in microbubbles for evaluation of NK cell mediated therapeutic intervention. Republished with permission from Chandrasekaran *et al.*, *Lab Chip* **14**, 1 (2019). Copyright Royal Society of Chemistry and Clearance Center, Inc.¹⁰² (f) Three-dimensional layout of drug assay displaying the layers for the gradient generator, barrier, and microwells. Reproduced with permission from Khoo *et al.*, *Sci. Adv.* **2**, 7 (2016). Copyright 2016 Authors, Creative Commons Attribution (CC BY) license.⁹³

control and careful geometric and size design of aggregated cells make microfluidics especially conducive for cellular experiments.

In treatment evaluation assays, microchambers can show individual cell/cluster responses to various concentrations of drugs and enable the continuous flow of media for short or long term cell culture. Bithi and Vanapalli¹⁰⁷ created cluster chambers to test chemotherapy drug doxorubicin on MCF-7 cells revealing that cells within a cluster have higher viability than their single-cell counterparts when exposed to the drug [Fig. 5(b)].¹⁰⁷ Desyatnik *et al.*⁹⁴ employed pneumatic microvalves integrated with cell culturing (up to 7 days) and parallel drug screening to test the chemosensitivity and resistance of MCF-7 and 293T cells [Fig. 5(c)]. Cells were seeded and cultured on the device, then as the valve blocking the drug chamber is opened, drugs flooded and diffused out into the cell chambers.⁹⁴ They also tested the sensitivity of MCF-7 cells to four different drugs at five different doses, each repeated in ten separate chambers.⁹⁴

Genetic and epigenetic characterizations have also been demonstrated via DNA methylation assays of liquid biopsy in a digital chip¹⁰⁸ as well as single-cell DNA sequencing using droplet microfluidics.¹⁰⁹ Sun *et al.*¹¹⁰ explored the spatial heterogeneity of CTCs within the circulatory system. They used Fluidigm single-cell qRT-PCR to investigate the dynamic expression of EMT-related genes in CTCs

during hematogeneous dissemination. They also studied the effects of high shear stress in blood vessels might induce the EMT phenotype in CTCs.¹¹⁰ Other organ-specific factors, such as interstitial pressure, oxygen gradient, and stromal cells, could also impact cell phenotype and tumor responses. These, therefore, may be implemented in microfluidic devices to accurately depict *in vivo* CTC clusters. For instance, abnormal fluid pressure also exists in the tumor as elevated interstitial fluid pressure. Shang *et al.*¹¹¹ investigated the interstitial pressure effect on the anticancer drug resistance in CTCs in a pressurized *in vitro* culture device for anticancer drug screening. They found that doxorubicin resistance can be increased by up to 2.5 times under 30 mm Hg due to the overexpression of an efflux transporter gene in human breast cancer cell lines.¹¹¹

Other characterization assays include assays to determine the adhesion strength of CTCs and, therefore, their metastatic potential. Mutlu *et al.*¹¹² reported an oscillatory inertial microfluidic system to investigate the cell-cell adhesion strength. They used a repeating fluidic force profile on suspended cell doublets without any biophysical modifications to the cell surface or physiological morphology. They analyzed doublets from a patient-derived breast cancer CTC line.¹¹² Another example uses a rectangular channel that allows high shear stresses to be generated under laminar conditions to quantify the

relationship between morphological characteristics and adhesion strength for well-spread cells.¹¹³ Long constriction channels can also be used for cells to transit in, and based on the friction coefficient, the cell adhesion strength may be obtained.¹¹⁴

Microfluidic systems have also been used to mimic 3D structural organization and dynamic microenvironment of CTCs for chemoresistance assays. Nath *et al.*¹¹⁵ built a perfusion model previously used to assess the effect of flow-induced shear stress on the genetic, molecular, and morphologic features of ovarian cancer in 3D culture over 7 days was modified to evaluate response to carboplatin treatment and photoimmunotherapy [Fig. 5(d)]. They showed the impact of flow-induced shear stress on resistance to carboplatin and modulation of EGFR (epidermal growth factor receptor) -mediated survival pathways in adherent 3D ovarian tumors.¹¹⁵ In another example, Chandrasekaran *et al.*¹⁰² showed the ability to culture tumor cells from surgical explants and studied their sensitivity to a therapeutic approach to target lymph node micrometastases.¹⁰² They developed spherical cavity culture systems that resemble anatomy of a deep cortical unit of a lymph to determine the effectiveness of liposome-based drug carrier therapies using NK cells conjugated with liposomes [Fig. 5(e)]. Wang *et al.*¹¹⁶ created a micropatterned tumor array, which described enabled detailed and dynamic characterization of CAR T cell trafficking toward tumor cell islands and targeting of tumor cells. Their assay allowed for the assessment of CAR T trafficking for immune-oncology research and preclinical assessments of cell-based immunotherapies.¹¹⁶ Khoo *et al.*⁹³ evaluated drug response of patient-derived CTCs of breast cancer patients throughout the time of treatment using their microwell system [Fig. 5(f)]. Their device was a multi-layer system that includes a channel barrier to prevent fluids with different concentrations from mixing at the cell culture region.⁹³ The presence of cancer cells was validated with FISH to identify cells with increased expression of breast cancer-associated markers, TOP2A and CCND1.⁹³ In a separate study, the same group also demonstrated a low-dose anti-inflammatory combinatorial treatment of doxorubicin and aspirin using the same device.¹¹⁷

With the success of single-cell analysis through microfluidics, the challenge now is to expand these applications to the study of rare cells and clusters. CTC clusters are of interest in the field due to their clinical significance. Downstream analysis of such clusters can provide insight into their genetic makeup, drug response, and resistance mechanisms. It is then important to keep patient-derived cells viable *ex vivo* for as long as possible to conduct such studies. With these in mind, it would be advantageous to integrate isolation systems with cell culture and analysis systems to speed up the post-processing and minimize manual handling.

VI. MICROFLUIDIC *IN VITRO* MODELS OF CTC CLUSTERS AND METASTASIS

Mechanical cues that are present in circulation such as flow, shear stress,¹¹⁸ and hydrostatic pressure¹¹¹ can impact CTC phenotype. High shear forces exerted on CTCs can lead to cell fragmentation and death,¹¹⁹ while intermediate shear forces promote extravasation.⁴⁶ Cancer cells in circulation may also have the potential to exploit these mechanical forces for their survival and successful seeding.⁴⁷ It has been shown that hypoxic conditions need to be sustained for primary CTCs to form clusters.^{93,120} In addition, the CTC microenvironment comprises unique features and facilitate interactions between immune cells

and CTCs.³⁴ Many microfluidic technologies can simulate the metastatic environment permitting accurate physiological and pathological investigations on cancer cell behavior, function, and viability.¹²¹ While molecular investigations may not be amenable in cell lines, physical and mechanical investigations with microfluidic devices could be possible.

In vitro studies of invasion and metastasis are especially conducive with microfluidic devices with the compartmentalization of microchambers with the use of valves. For instance, both intravasation and extravasation studies were conducted in an integrated microfluidic chip to study the metastatic cascade.¹²² Shin *et al.*¹²² created a device that consists of an intravasation chamber for a 3D culture of cancer cells using a Matrigel matrix and an extravasation chamber [Fig. 6(a)].¹²² Their device demonstrated the detection of metastasized cancer cells by adhesion molecules expressed by epithelial cells.¹²² In another study, Zhang *et al.*¹²³ created a microfluidic device that is representative of the principal components of biological blood vessels, including vessel cavity, endothelium, and perivascular matrix [Fig. 6(b)]. Their goal was to study the transendothelial invasion of salivary gland adenoid cystic carcinoma cell aggregates under chemokine stimulation.¹²³ A physiologically relevant model of transendothelial extravasation was also created by Chen *et al.*¹²⁴ using a microfluidic platform that incorporates a self-organized 3D microvascular network [Fig. 6(c)]. The device is coupled with the capability for live tracking of single-cell and cell cluster extravasation events, allowing both tumor and endothelial morphological dynamics to be observed. They found that tumor cell transendothelial migration efficiency was higher for cell clusters compared to single cells.¹²⁴ In another example, Sung *et al.*¹²⁵ modeled the transition of ductal carcinoma *in situ* (DCIS) into invasive ductal carcinoma (IDC) in a compartmentalized co-culture system to facilitate the observation of one cell type independently and of distance-dependent effects [Fig. 6(d)].¹²⁵

Microfluidic systems permit higher complexity compared to other conventional 3D culture systems, which allow for the investigations of key roles that other cells or signaling factors play during cancer progression. McCutcheon *et al.*¹²⁶ used a microfluidic platform to distinguish collective migration of neuroclusters from that of individual cells in response to controlled concentration profiles of stromal-derived growth factor (SDF-1). With the ability to precisely form heterotypic cell clusters, Fatsis-Kavalopoulos *et al.*⁹⁵ conducted paracrine signaling studies in a controlled cell assembly generator. They were able to spatially resolve and analyze paracrine effects of β -cell ATP (adenosine triphosphate) release on Ca^{2+} dynamics in three to four breast cancer cells.⁹⁵ Furthermore, careful design and the addition of different cell types, chemokines, and extracellular matrix (ECM) components can model more complex mechanisms. Lee *et al.*¹²⁷ investigated the immunosuppressive role of monocytes and PD-L1/PD-1 signaling on targeting of hepatitis B virus (HBV)-specific T cell receptor-redirected (TCR) T cells using a 3D static microfluidic model [Fig. 6(e)]. They created a 3D co-culture system of target HepG2 cell aggregates, HBV specific TCR T cells, and monocytes within a microfluidic device, where monocytes were suspended together with target cell aggregates in collagen gel.¹²⁷

One of the main features of microfluidic devices is the micro-sized channels and chambers that offer flexibility in design. Rizvi *et al.*¹⁹ compared static and flow conditions on ovarian cancer cells and clusters to evaluate the effects of flow on the growth of 3D ovarian micro-nodules. They found that compared with nonflow cultures, 3D

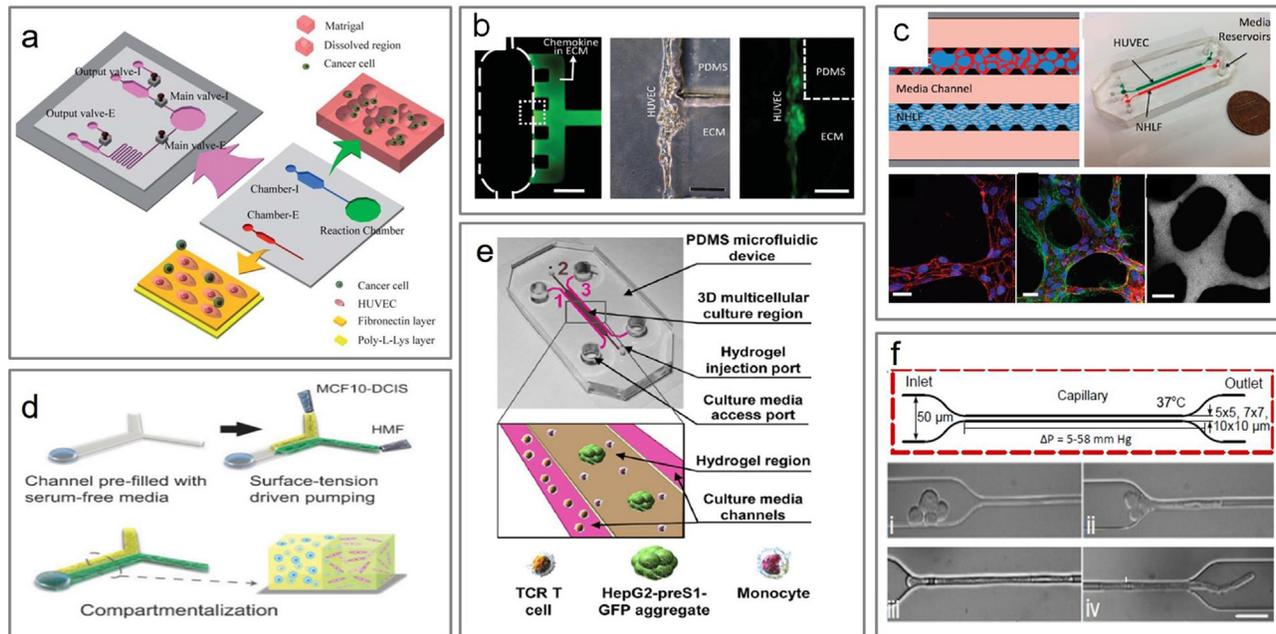


FIG. 6. Modeling the metastatic cascade in microfluidics. (a) Integrated cell-based microfluidic chip and the simulation of intra- and extravasation. Republished with permission from Shin *et al.*, *Lab Chip* **11**, 22 (2011). Copyright Royal Society of Chemistry and Clearance Center, Inc.¹²² (b) Constructed blood vessel model where PDMS pillars between side channels form the scaffold for ECM. Republished with permission from Zhang *et al.*, *Lab Chip* **12**, 2837 (2012). Copyright Royal Society of Chemistry and Clearance Center, Inc.¹²³ (c) A microfluidic microvascular network platform for studying tumor cell extravasation. Reproduced with permission from Chen *et al.*, *Integr. Biol.* **5**, 1262 (2013).¹²⁴ Copyright 2013 Oxford University Press. (d) 3D compartmentalization and the intravasation of MCF-DCIS cells. Reproduced with permission from Sung *et al.*, *Integr. Biol.* **3**, 439–450 (2011).¹²⁵ Copyright 2013 Oxford University Press. (e) A microfluidic model of the multicellular tumor microenvironment with a middle hydrogel channel. Reproduced with permission from Lee *et al.*, *Front. Immunol.* **9**, 416, (2018). Copyright 2018 Authors, licensed under a Creative Commons Attribution (CC BY) license.¹²⁷ (f) Constriction device for cluster migration. Reproduced with permission from Au *et al.*, *Proc. Natl. Acad. Sci.* **113**, 18 (2016). Copyright 2016 Authors, licensed under a Creative Commons Attribution (CC BY) license.¹²⁸

ovarian cancer nodules grown under flow exhibited morphological features indicative of increased EMT and different expression levels of EGFR, E-cadherin, CDC2, and p27Kip1.¹⁹ In another instance, Au *et al.*¹²⁸ introduced a microfluidic device that mimicked constrictions of the human capillary using $10 \times 10\text{-}\mu\text{m}^2$ cross-sectional channels [Fig. 6(f)]. They found that $>90\%$ of CTC clusters contain up to 20 cells successfully traversed $5\text{--}10\text{-}\mu\text{m}$ constrictions even in whole blood. The CTC clusters effectively reorganized into single-file geometries, which substantially reduced their hydrodynamic resistances.¹²⁸ King *et al.*¹⁰¹ explored the hemodynamic force effects on cancer cell cluster transport. They used microrenathane microtubes with an inner diameter of $300\text{-}\mu\text{m}$ functionalized with E-selectin to mimic blood vessels.¹⁰¹ They were able to simulate CTC-endothelial cell interactions by quantifying rolling velocity and displacement of the cell line aggregates on E-selectin coated surfaces.

Microfluidics has proven to be very useful in creating *in vitro* models used to study the metastatic cascade due the ability to fully capture key features of the immune microenvironment that occur *in vivo*, especially during the metastatic spread.¹²⁹ In addition, physical forces that CTCs experience during their transit in the blood can be precisely mimicked in microchannels. Microfluidic systems also play a role in downstream cellular investigations and allow for high-resolution imaging. Furthermore, *in vitro* systems allow for the use of human-derived cells exclusively without the confounding effects of animal host cells observed in animal models.¹²⁹

VII. SUMMARY AND OUTLOOK

As microfluidic devices are gaining popularity for cellular manipulation and analysis, these technologies are now also applied in drug screening and cellular characterization. Microfluidic isolation of cells and clusters, including label-free and immunoselection approaches, based on either passive or active methods, have progressed in efficiency and throughput paving the way for liquid biopsy as a relatively easy and minimally invasive way to obtain primary cells for disease monitoring. Current microfluidic cluster isolation methods can achieve high efficiency (up to $>99\%$)⁵⁶ and viability ($>90\%$).¹²⁸ Compared with passive isolation methods, the active methods exhibit higher efficiency but slightly lower cell viability. Active methods are also more complex in operation and would overall need improvement in viability, cost, and simplicity. The ability to isolate CTC clusters is a significant step toward tumor cell characterization and modeling. However, although microfluidic isolation systems have become more sophisticated over the past few years, the rarity of primary cell clusters restricts the extensive characterization and screening of primary cells and clusters. There must then be an improvement in microfluidic systems in areas, including cell retention, viability, sensitivity, and physiological modeling.

Downstream analysis especially at the single-cell level remains to be a challenge. For instance, enzyme-linked immunosorbent assays (ELISA), PCR, western blots, and mass spectrometry require sufficient

number of recovered cells per test since low cell densities may not be enough for accurate molecular analysis. With onset low viability of CTCs, there could be even lesser viable cells available for such analyses.⁴² The cell capture rates of different single-cell RNA-seq methods can be as low as >1% or go up to 62%.¹³⁰ In this realm, sample loss needs to be significantly minimized. Much of the cell loss can be attributed to manual handling of samples such as the transfer from one device to another. There are currently limited options in which small cell numbers can be handled with minimal manual interference. Thus, these issues would need to be addressed perhaps with the development of streamlined microfluidic methods.^{50,61} For methods of viability assessment, fluorescence labeling is used to track the viability of cells; however, these signals diminish overtime and so the long-term monitoring of cells may be difficult. Other ways of indicating cell viability would be to determine the metabolic and proliferation rates of cells through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), cell cycle, or DNA synthesis assays. Such assays may need to be incorporated into microfluidic platforms to study CTCs.

On the other hand, with such low cell densities, there is then a need for the improvement on the detection sensitivity of many common molecular analysis methods to achieve true single cell analysis. The varied levels of surface marker expression, for example, can cause labeling methods to be inconsistent. Physical detection or a combination of the label-based and physical methods may, therefore, help mitigate the problem during separation.¹ At the same time, the expansion of CTCs and CTC clusters can take a long time and, thus, necessitates the development of new methods for rapid expansion of patient-derived tumor cells. Even so, personalized medicine still holds much promise with the microfluidic approach, where processing time could be significantly improved with automation and the integration of upstream methods (i.e., isolation, cell expansion/culture, etc.) with downstream analysis.¹³¹ Routine screening for longitudinal disease prognosis with microfluidics can provide clinicians with patient-specific information and, therefore, guide early personalized therapeutic interventions as well as monitor disease state. With the development of more droplet microfluidic systems, single cell analysis tremendously advanced through use of nano- to picoliter droplets and barcoding.^{109,132,133}

Despite much of the advancements and increased complexity in many microfluidic systems, there are still many challenges affecting performance of these devices in CTC and clusters analysis. For one, a general shortcoming of *in vitro* systems is their inability to fully recreate or replace the *in vivo*. Cell clusters formed *in vitro* may exhibit differences in phenotype and gene expression compared to primary tumors which are highly heterogeneous. The use of cancer cell lines may only provide an alternative and be used as surrogates to complement studies of primary cells.³¹ Moreover, several factors contribute to the metastatic cascade and extravasation and, thus, influence CTC seeding patterns. The growth and metastasis of tumor are not only based on the cancer cell simply but also rely on cancer-associated fibroblasts and immune cells within tumors and other supporting tissue that surround the tumors.

Nevertheless, these shortcomings may be diminished in microfluidic systems that can recapitulate the tumor microenvironment through the introduction of different cell types and the application of mechanical stresses and/or chemical cues. Because of the ability to control biological and physical factors, microfluidic systems have the

potential to be more comprehensive and accurate models. Another main limitation in microfluidics is that it may require specialized training in operation as well as fabrication, which is due to the lack of established standards in device development. In order to translate these systems into the clinical setting, operation should become simpler and user-friendly. Drug screening platforms also need to be high-throughput comparable to standard well plates used in commercial screening. Because of the limitations brought on by low cell densities, *in vitro* CTC cluster modeling may be necessary to develop better systems for CTC cluster isolation, culture, and analysis.

Microfluidics can also be used for development of *in vitro* models of CTC clusters due to superior spatial control needed to mimic the cellular microenvironment. With the capability to permit precise control of flow, mechano-transduction pathways involved in metastasis may be investigated more closely to identify the biomechanical cues that may promote extravasation, migration, and eventual seeding of tumor cells.¹¹⁸ Gravity-based sedimentation into microwells, cell-specific capture through antibody-coated surfaces, or entrapment in microdroplets in microfluidic devices is often used. These approaches allow them to form clusters on-chip while precisely controlling the cell number and position. The wide size-range of cluster models from less than ten cells to thousand cells is achieved to meet various application requirements in personalized medicine. Microwell size and geometries may be tunable by using laser ablation or 3D printing of microwell molds to achieve precise formation of individual cell clusters, minimize cell loss, and prevent cluster dissociation. Additionally, microfluidics offers advantages of small sample consumption, high throughput, and low cost. These systems also enable the visualization of cells at high resolution due to the short distance from the biological samples in the device to the microscope objective. However, there are limitations in imaging larger-sized cell aggregates when the required working distance becomes too large.

Overall, even with the growing research in the field, much has yet to be learned regarding the prognostic value and potential role of CTC clusters in personalized medicine and drug development. Thus, there are opportunities in the development of *in vitro* models that can fully encompass the intricacies of the metastatic cascade. This may be done through the combination of multiple factors such as biochemical signaling and mechanical stimulation or done through the decoupling of such factors that are usually confounding in *in vivo* models. In addition, future opportunities in device development for CTC cluster studies would be in the integration of multiple procedures such as isolation, culture, and downstream analysis in a single device.⁸⁶ This can hopefully provide ease in the processing of biological samples and ultimately offer insight into drug screening and development of personalized patient cancer treatments.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Ethics Approval

Ethics approval is not required.

Author Contributions

Celine Macaraniag: Conceptualization (supporting); Visualization (lead); Writing—original draft (lead). **Qiyue Luan:** Writing—original draft (supporting); Writing—review and editing (supporting). **Jian Zhou:** Conceptualization (equal); Investigation (equal); Writing—review and editing (equal). **Ian Papautsky:** Conceptualization (equal); Funding acquisition (lead); Supervision (lead); Writing—review and editing (lead).

DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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