Trends in Biotechnology

CellPress

Review

Single-cell analysis of circulating tumour cells: enabling technologies and clinical applications

Payar Radfar,[1,11] Hamidreza Aboulkheyr Es,[1,11] Rob Salomon,[2,3] Arutha Kulasinghe,[4,5] Naveen Ramalingam,[6] Ehsan Sarafraz-Yazdi,[7] Jean Paul Thiery,[8,9] and Majid Ebrahimi Warkiani [1,2,8,10,*]

Multimodal analysis of circulating tumour cells (CTCs) has the potential to provide remarkable insight for cancer development and metastasis. CTCs and CTC clusters investigation using single-cell analysis, enables researchers to gain crucial information on metastatic mechanisms and the genomic alterations responsible for drug resistance, empowering treatment, and management of cancer. Despite a plethora of CTC isolation technologies, careful attention to the strengths and weaknesses of each method should be considered in order to isolate these rare cells. Here, we provide an overview of cutting-edge technologies used for single-cell isolation and analysis of CTCs. Additionally, we highlight the biological features, clinical application, and the therapeutic potential of CTCs and CTC clusters using single-cell analysis platforms for cancer management.

Highlights

In-depth characterisation of circulating tumour cells (CTCs) has shown promise in diagnosis and management of cancer patients in a noninvasive manner. However, rarity of CTCs poses critical challenges for isolating and analysing them.

CTC analysis provides insights into tumour heterogeneity beyond genomic aberrations that are not found in circulating tumour DNA (ctDNA).

Single-cell molecular analysis of CTCs offers a new prognostic approach to identification of targeted therapy resistance mechanisms.

Choice of technology for isolation and analysis of CTCs mainly depends on cell-loss, study cost per CTC, and workflow complexity.

Current technologies suffer from high analysis costs and cell-loss during handling of the low number of CTCs. Additionally, different physical and biological features of CTC clusters often lead to difficulties in analysing them via current commercial platforms.

Importance of analysing CTCs

Cancer cells are heterogeneous, and this inherent property appears to be one of the main challenges in shifting the current paradigm towards improving cancer treatment. Among the hallmarks of cancer, metastasis leads to >90% of cancer-related deaths [1]. In recent years, our understanding of the molecular alterations that drive tumour progression and metastasis have improved, which has revolutionised the clinical management of solid tumours towards a more personalised approach. Identifying genomic drivers of cancer initiation and progression has led to the clinical development of a new generation of therapeutic agents, known as targeted therapies. These drugs often target gene products controlling cancer cell proliferation and other survival mechanisms. However, these targeted therapies often lead to therapeutic resistance by the development of mutations in oncogenes or activation of bypass signalling pathways [2]. The longitudinal monitoring of patients' response to a targeted therapy using repeated tissue biopsies is invasive and often impossible due to the size and location of tumours.

An alternative approach involves the analysis of **CTCs** (see Glossary), including single cells and clusters of cells. CTCs refer to the population of cancer cells in the blood circulation, released from primary or metastatic tumours (Figure 1) [3]. While it has been proven that CTCs have a short half-life [4], it is clear that a small number of these can eventually initiate new metastases [5–8]. The genome-wide single-cell RNA sequencing (RNA-seq) and DNA-sequencing performed on CTCs have provided new insights into CTC heterogeneity and mechanisms of therapeutic resistance to targeted therapies among patients with solid tumours [9]. A significant number of reviews has been published around CTC analysis, discussing the clinical importance and implications of CTCs [10–12]; however, the technical consideration of CTC and CTC cluster analysis have not yet been discussed. In this review article, we describe the recent advancements of technologies developed for single-cell analysis, comprehensively discussing the advantages and disadvantages of each approach for analysis of individual and clustered CTCs. Additionally, we

[1]School of Biomedical Engineering, University of Technology Sydney, Sydney, Australia
[2]Institute for Biomedical Materials and Devices (IBMD), Faculty of Science, University of Technology Sydney, Sydney, NSW 2007, Australia
[3]ACRF Child Cancer Liquid Biopsy Program, Children's Cancer Institute, Sydney, Australia
[4]University of Queensland Diamantina Institute, The University of Queensland, Brisbane, Queensland, Australia
[5]Translational Research Institute, Brisbane, Australia
[6]Fluidigm Corporation, South San Francisco, California 94080, USA
[7]NomoCan Pharmaceuticals, New York Blood Centre, New York, NY 10065, USA

highlight the clinical application of single-CTC and CTC cluster analysis in monitoring targeted therapy response in cancer patients towards personalised medicine.

Cellular and molecular features of CTCs

Phenotypic variation amongst CTCs suggests that specific subpopulations of CTCs exist, and this variation may impart differential metastatic potential [9,13]. Numerous studies have discovered the link between **epithelial–mesenchymal transition (EMT)** and the acquisition of stemness properties in various cancers [14,15]. The expression of EMT-related and stem cell markers, including but not limited to CD44 and vimentin, has been identified in a subpopulation of CTCs with the mesenchymal state indicating the existence of cellular heterogeneity among CTCs [13,16,17]. For instance, both early and metastatic stages of breast carcinoma show an increased number of CTCs with a mesenchymal phenotype [2]. In pancreatic ductal carcinoma (PDAC), single-cell RNA-seq analysis of CTCs identified a loss of epithelial markers E-cadherin and mucin-1 compared with the primary tumour. Remarkably, the expression of pancreatic stem cells markers, ALDH1A1 and ALDH1A2 in CTCs does not correlate with EMT status, suggesting that EMT and stemness may not be linked in this pancreatic cancer model and thus may follow a tissue-dependent pattern [18]. In addition to these findings, several studies have highlighted the role of CTCs in presenting an immune escape mechanism from the body's immune surveillance by the expression of the immune checkpoint protein programmed death-ligand 1 (PD-L1) detected on both single and clustered CTCs from various types of cancer, including lung and head and neck carcinoma [19–22]. These studies highlight how CTC PD-L1 expression may provide a proxy for determining tumour PD-L1 expression, and a measurement for predicting immunotherapy response in these cancer types [22,23].

The number of CTCs in the blood depends on different factors, such as cancer type and disease status. However, estimates suggest that CTC counts often range between 1 and 100 for every 10^7 white blood cells [24]. While detecting CTCs is challenging due to their rarity, phenotypic (i.e., size) and biological attributes (i.e., cell surface protein expression) can be utilised to enrich and eventually isolate CTCs among other peripheral blood cells.

Box 1 provides further information on CTC enrichment approaches. Although each enrichment technique has its own advantages and shortcomings, high contamination of background cells in the enriched sample and false depletion of target cells remain as the main challenges during the CTC enrichment process [25]. The high contamination of unwanted cells in the CTC enriched samples leads to challenges for analysis of CTCs [26]. Thus, often an additional step of single-cell isolation is required to study CTCs individually [27].

Understanding tumour heterogeneity using genomic analysis of CTCs

While CTC enumeration has prognostic value, molecular characterisation and functional testing of captured CTCs can lead to a better understanding of the disease state and potential treatment options [28]. CTCs are often heterogeneous and understanding them at single-cell resolution reveals unique information that is normally masked by bulk/pooled analysis of the samples [11]. Recent studies on single CTCs discovered key insights into the clonal and dynamic evolution of CTCs in response to therapies [29]. For instance, a diagnostic leukapheresis approach identified tumour heterogeneity by analysing CTCs derived from prostate cancer patients [30]. This method allows the analysis of hundreds of CTCs and the identification of sub-clonal **copy-number variations (CNVs)** that were not easily distinguished in bulk analyses of tumour biopsies [30]. In another example, in multiple myeloma cancer patients, similar clonal profiles were observed between bone-marrow-derived cancer cells and isolated CTCs, with discordances restricted to subclonal mutations [31]. It has been found that the mutation spectrum and mutation burden

[8]Centre of Biomedical Engineering, Sechenov University, Moscow, 119991, Russia
[9]Bioland Laboratory, Guangzhou Regenerative Medicine and Health, Guangdong Laboratory, Guangzhou, 510320, China
[10]Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia
[11]These authors contributed equally

*Correspondence:
majid.warkiani@uts.edu.au
(M.E. Warkiani).

of CTCs and other overt metastases closely resemble regions of the primary tumour known as the metastasis-initiating area [18].

However, the benefit of molecular analysis of CTCs for the study of tumour heterogeneity remains controversial due to the low number of isolated CTCs [11]. Various groups have attempted to address this issue with the use of pooled CTC samples for molecular analysis. Recently, a comprehensive CTC profiling of a panel of 130 genes was performed using individual and pooled CTCs derived from metastatic breast cancer patients [32]. Comparing their metastatic tissue counterparts revealed 85% concordance between individual and pooled samples in at least one or more recurrent somatic mutations and copy number aberration [32]. The presence or absence of CTCs can be further used to unravel the molecular pathways activated or altered during the tumour and metastasis evolution process. For example, distinct gene expression signatures have been found for breast and lung carcinoma from patients with and without CTCs in the blood or **disseminated tumour cells (DTCs)** in the bone marrow [33]. Indeed, profiling CTCs from breast cancer patients at the single-cell level showed remarkable intrapatient heterogeneity in the expression of cancer-associated genes [34,35].

CTC single-cell isolation techniques

While CTCs were traditionally analysed through routine imaging that allows for CTC enumeration using a handful of markers, the emergence of enrichment and single-cell isolation technologies have allowed for downstream analysis of CTC with greater depth of characterisation, which provides crucial information of the primary tumour [25]. However, low recovery rate of CTCs and high contamination of background cells in the enriched sample often poses technical difficulties for molecular and functional characterisation of CTCs [11,36]. Moreover, bulk analysis obscures key information and tends to mask the level of heterogeneity among single CTCs [11,37]. Thus, use of single-cell analysis technologies can enhance the analysis of CTCs and may identify the potential clinical use of CTCs as a cancer biomarker.

In this section, commonly used single-cell analysis platforms for characterising CTCs are discussed. Figure 2 illustrates the conventional and microengineered single-cell technologies. The commercial implementation of these approaches is shown in Figure 3, and a technical comparison of each technique is provided in Table 1. Furthermore, Table 2 contains detailed information on studies discussed in this section. Last, Box 2 provides detailed information on types of single-cell analysis often performed post CTC isolation.

Limited dilution

Limited dilution, also known as serial dilution, is a simple and cost-effective method for isolation of single cells by dispensing between 0.3 and 0.5 cells per dispense volume. As the distribution follows **Poisson distribution** probability, this approach results in a high number of empty wells but critically minimises the multioccupancy rate [38]. This method can be achieved using a common handheld pipette or pipetting robots and hence is a low-cost approach. Despite the accessibility, this approach is less favourable for isolation of CTCs at the single cell level given the rarity of these cells and large number of wells that would be required [39]. It should also be noted that modern high-throughput single-cell genomics instruments such as droplet and nanowell systems use limiting dilution to minimise doublet rates during cell encapsulation.

Micropipetting and micromanipulation

Another approach for the manual isolation of single CTCs from an enriched sample is using a micropipette made from an ultrathin glass capillary. In this approach, the enriched sample is analysed under a microscope, and the cells of interest are identified often based on fluorescent

Glossary

Alectinib: an oral drug that blocks the activity of anaplastic lymphoma kinase (ALK) and is used to treat non-small-cell lung cancer harbouring activating mutations of this gene.
Apheresis: a medical procedure allowing the fractionation of whole blood to isolate different blood cell types before being reintroduced into the body.
Circulating tumour cells (CTCs): cancer cells that disseminate from primary tumour sites and enter the vasculature system.
Copy-number variations (CNVs): occurs when the number of copies of a specific gene change from the normal two copies.
Crizotinib: anticancer drug acting as an ALK and c-ros oncogene 1 (ROS1) inhibitor and is used to treat non-small-cell lung cancer harbouring activating mutations of these genes.
Disseminated tumour cells (DTCs): cancer cells residing in a distant organ such as bone marrow, following their dissemination from the tumour.
Epithelial–mesenchymal transition (EMT): a process that allows an epithelial cell to transform to a mesenchymal cell phenotype.
Insertion or deletion (InDel): mutation in which extra base pairs are inserted in the genome (insertion) or some DNA sequences are deleted (deletion).
Magnetic-activated cell sorting (MACS): a method for separation of various cell populations depending on their surface antigens.
Microfluidics: science of handling tiny volumes of liquid in micro/nanometre-sized channels.
Poisson distribution: the probability distribution that is used to show the likelihood that an event will occur within a certain time.
Single-nucleotide variants (SNVs): occur when a single nucleotide is changed in the DNA sequence.

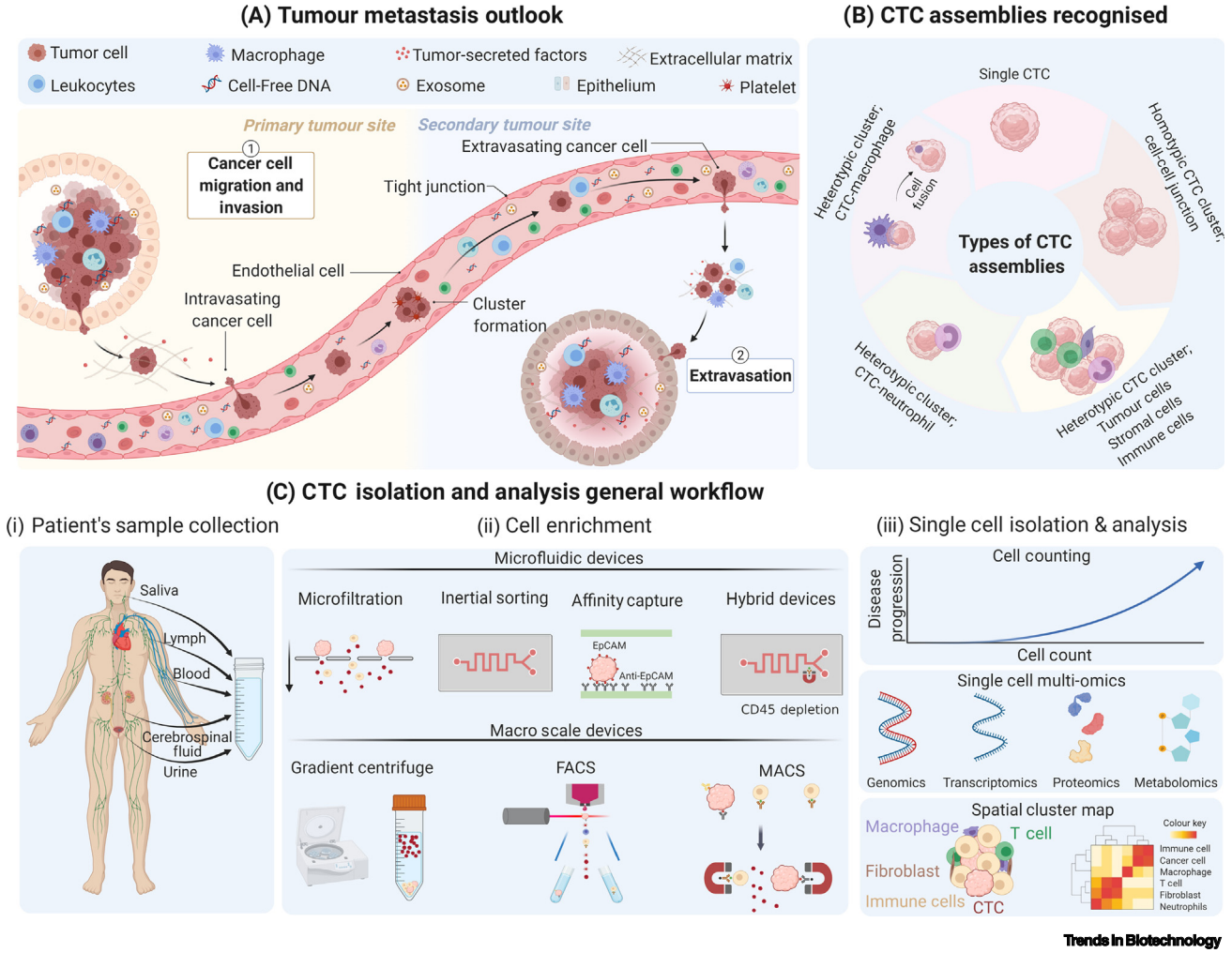

Figure 1. Schematic illustration of the role of circulating tumour cells (CTCs) in cancer metastasis in various forms and the current workflow for analysis of them.

For a Figure360 author presentation of Figure 1, see the legend at https://doi.org/10.1016/j.tibtech.2022.02.004

(A) Overall outlook of tumour progression with key biological steps of metastasis through intravasation, circulation, and extravasation. (B) Various CTC assemblies recognised as single cells and clusters [105]. (C) Typical workflow for isolation of CTCs including sample collection, CTC enrichment, and single-cell characterisation. Created with Biorender. Abbreviations: FACS, fluorescence-activated cell soring; MACS, magnetic-activated cell sorting.

labelling and morphology. Then, an ultrathin glass micropipette approaches the cell of interest and is manually aspirated (e.g., mouth pipetting), which then is deposited into a collection tube [38]. The major drawbacks of single-cell isolation through manual micropipetting are the low throughput, labour intensiveness, and reliance on operator's skills [40]. For instance, in a study by Xu and coworkers, micropipetting has been used to isolate and analyse CTCs in blood samples from 20 early-stage lung cancer patients before and after one cycle of treatment to reveal detailed genetic variations of the CTCs [41].

Micromanipulators, as opposed to micropipettes, are typically semiautomated single-cell isolation platforms that consist of an inverted microscope paired with micropipettes that are controlled by a mechanical interface. Micropipettes are ultra-thin capillary glasses, connected to an aspiration and dispensation unit with capability of handling liquid down to nanolitre scale [38].

Box 1. CTC enrichment methods

Enrichment and detection techniques influence CTC enumeration as well as downstream assessment methods. There is significant variability of results, depending on blood volume, sample preparation protocols, isolation methods, and CTC classification criteria. Therefore, careful analysis considering the methodologies and parameters applied in each study is essential, particularly for single-cell analysis. Current CTC enrichment methods can be mainly categorised based on their functionality into immunoaffinity and physical-based approaches.

Immunoaffinity-based methods utilise the surface biomarkers of cells for separation of CTCs, through a positive enrichment of cancer cells using epithelial markers such as EpCAM, or negative depletion of leukocytes using haematopoietic cell markers such as CD45 [92]. The most-used immunoaffinity cell sorting technique is FACS, by which cells are stained and passed through a fluorescent detection system. The cells of interest are sorted into one or more tubes based on their fluorescent tag. Magnetic-based separation methods (i.e., **magnetic-activated cell sorting**; **MACS**) are also developed by which the cells are tagged with magnetic beads and then separated using a magnet. The only FDA approved technology for CTC isolation, that is, CellSearch, utilises EpCAM-coated beads for CTC isolation. The main challenges of these methods are the low-throughput and inability to capture cells with low or no expression of the specific surface biomarker, including CTCs that undergo dedifferentiation, losing their epithelial markers such as in EMT. This phenotypic alteration significantly reduces the overall CTC capture efficiency [93,94].

Physical-based approaches utilise the phenotypic attributes of CTCs for enrichment, including different density, size, and deformability compared with other peripheral blood cells [11]. Among physical-based enrichment methods, microfilters (e.g., isolation by size of tumour cells; ISET) and inertial microfluidics have been widely used for isolation and purification of CTCs from a wide range of body fluids. One of the main advantages of physical-based methods is the enrichment of intact and viable cells that are suitable for downstream single-cell analysis. Physical separation techniques can also result in a much shorter enrichment time and are expected to cost less without biochemical modifications. However, background contamination by larger leukocytes and loss of smaller CTCs remains an outstanding challenge for these systems [8].

Recently, more complicated technologies have been developed to separate CTCs by benefiting from both immunoaffinity and physical based approaches integrated in one device. These platforms are often referred as hybrid devices and can minimise the contamination of background cells [95]. However, hybrid technologies are often complex – that is, difficult to manufacture and operate. Thus, they have not yet been clinically implemented [96].

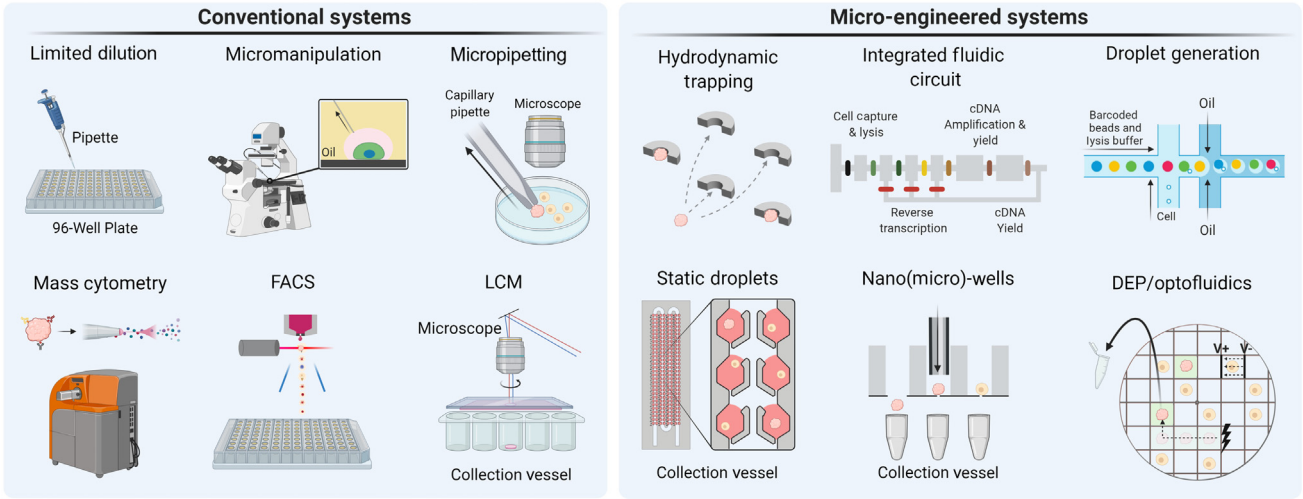

Figure 2. Single-cell isolation techniques discussed in this review are primarily categorised into conventional and microengineering devices. The conventional systems include; (i) limited dilution using a handheld laboratory pipette, (ii) micromanipulation using a micropipette on a robotic arm to allow precise manipulation/handling of liquid, (iii) micropipetting using a thin capillary pipette under a microscope, (iv) mass cytometry that determines cellular properties via antibodies labelled with metal ion tags, (v) fluorescence-activated cell sorting (FACS) that uses cell surface biomarkers to isolate and deposit single cells into wells, and (vi) laser capture microdissection (LCM) that takes advantage of the energy of the laser beam to detach the cell of interest from a slide. Microengineering devices include; (i) hydrodynamic traps that utilise fluidic resistances to trap cells, (ii) integrated fluidic circuits that feature digital valves that handle cells for analysis, (iii) droplet generation that encapsulates cells and a barcoded bead through liquid-in-oil segmentations, (iv) static droplets that fractionate liquid using capillary forces, (v) nano (micro)-wells that isolate single cells inside nanolitre-sized wells that can be used to isolate cells, and (vi) dielectrophoresis (DEP) and optofluidic devices that combine microfluidics with microelectronics and optics, respectively, to precisely manipulate cells of interest. Created with Biorender.

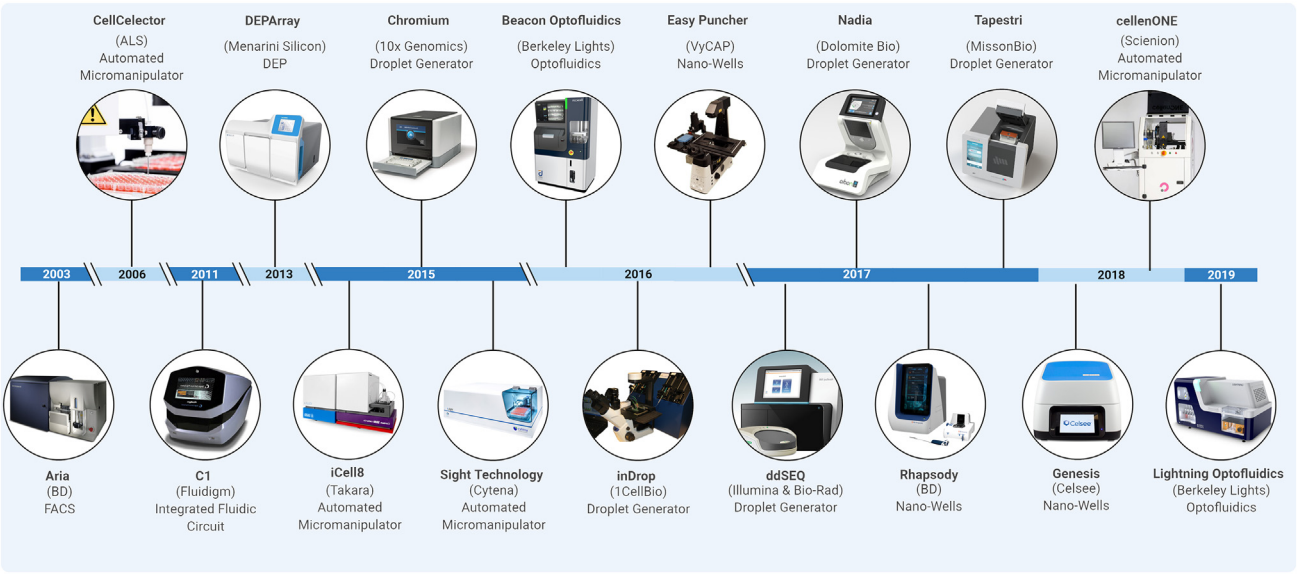

Figure 3. Timeline of commercial single-cell products. With advancements in technology, single-cell isolation and analysis platforms have been emerging since early 2006. Different technologies can primarily be categorised based on functionality into: automated micromanipulation, fluorescence-activated cell sorting (FACS), nanowell systems, droplet generators, dielectrophoresis and optofluidics. Created with Biorender.

In this technique, the CTC enriched sample is often provided as a suspension in a dish or centrifuged on a slide, where the operator identifies the cell of interest using typical CTC surface biomarkers; for example, epithelial cell adhesion molecule (EpCAM). The micropipette is driven to the proximity of the cell and is aspirated via a suction force for consequent transfer of the cells to a collection vessel [42]. As an example, using this approach, Lohr and colleagues reported an integrated process to isolate, qualify and sequence whole exomes of individual CTCs where they identified ~70% mutation similarity of CTCs with the original tissue in prostate cancer patients [18]. Despite the advantages of this approach, including high-precision liquid handling and low sample loss, micromanipulation of single cells is a time-consuming, labour-intensive method and can cause damage to the cells which limits the applicability of this approach in clinical settings [40].

To overcome these limitations, commercial products have been developed to automate the cell detection and isolation process within a short time frame (Figure 3). In a study by Gkountela and coworkers, DNA methylation profiles of single CTC and CTC clusters from 43 breast cancer patients and 13 mouse models were analysed to understand the link between CTC clustering and specific DNA methylation changes which can promote stemness and metastasis [43]. In a similar study, Reinhardt and colleagues combined a microfluidic enrichment method named diagnostic leukapheresis with an automated micromanipulator followed by a subsequent single-cell transcriptome profiling of CTCs from seven breast cancer patients [44]. Despite the advantages of automated micromanipulators for identifying, isolating, and transferring cells based on their morphology and biomarkers in a labour-free and nonintensive way, this method still suffers from high setup costs, system complexity, and low transfer efficiency while handling adhesive cells.

Laser-capture microdissection

Laser-capture microdissection (LCM) is a tissue capture technique to isolate single cells from mostly solid tissue slices [45]. Alternatively, this technique has been adopted for isolation of

Table 1. Detailed comparison between most-used single-cell isolation techniques and how they relate when dealing with CTC analysis

	Micromanipulation	FACS	Droplet generators	Nanowells	DEP and optofluidics	Limited dilution	LCM
Capture efficiency	High	Moderate–high	Moderate	Moderate–high	High	Low	High
Doublet rate	Low Dependent on operator's skills and/or concentration of cells on the imaging slide	Low Related to sort mask	Low–moderate Related to the loading concentration	Low–moderate Related to the loading concentration	Low Related to the loading concentration	Low Related to the loading concentration	Low–moderate Related to the loading concentration
Throughput	Low	High	Moderate	Moderate–high	Moderate	Extremely low	Low
Upfront cell selection	Yes	Yes	No	No	Yes	No	Yes
Starting amount	Hundreds–thousands	Tens of thousands–millions	Five hundred–tens of thousands	Five hundred–tens of thousands	Up to tens of thousands	Hundreds	Hundreds
Laboratory skills	Moderate	High	Moderate–high	Moderate–high	Moderate–high	Low	Moderate–high
Cell stress	Low	Moderate–high	Moderate	Low	Moderate	Low	High
Equipment costs	Moderate–high	High	Moderate–high	Low	Extremely high	Extremely low	High
Commercial products	CellCelector (ALS) Eppendorf micromanipulators SIGHT – Families (Cytena) cellenONE (Scienion) iCell8 (Takara)	FACSAria (BD Sciences)	GEM technology (10xGenomics) ddSEQ (Illumina & Bio-Rad) Tapestri Platform (Mission Bio) Nadia (Dolomite Bio) InDrop (1CellBio)	Rhapsody (BD Sciences) C1 (Fluidigm) Easy Puncher (VyCAP) Celsee	DEPArray (Menarini Silicon Biosystems) Lightning Optofluidic (Berkeley Lights) Beacon Optofluidic (Berkeley Lights)	Standard laboratory pipettes	Arcturus XT (Thermo Fisher) LMD6&7 (Leica Microsystems) CellCut (MMI)
Recommendations	Often suitable after an initial enrichment with a great flexibility for different downstream analysis. Higher throughput is achieved via automated systems. Ability to select individual cells that can significantly lower the analysis costs.	Suitable for second purification and samples with high contamination. Using FACS for single-cell isolation often becomes challenging when dealing with low sample input such as CTCs.	Not recommended for pure low load CTC samples. Not flexible with different analysis types. For CTC analysis, sample pooling is required which will increase the analysis costs.	Vary in range from simple to complex systems and are mostly cost-effective. They are more flexible with downstream analyses. Each nanowell can be used for isolation and/or reaction chamber for different analysis.	High control in cell handling and great choice for single CTC isolation in an automated way. However, they are complex and have an extremely high setup and operational costs.	Limited dilution and LCM approaches are less commonly used for CTC isolation due to their technological limitations including, low throughput and labour intensiveness.	

Table 2. Summary of key studies on CTCs via different single-cell isolation platforms

Study no.	Single-cell isolation technology	Single-cell isolation feature	CTC enrichment technique	Cancer type/organ	Significance and outcomes	Refs
1	Micropipetting	Surface biomarkers	Deterministic lateral displacement and immunomagnetic – white blood cell (WBC) depletion	Signet ring cell carcinoma and adenocarcinoma	Xu and colleagues developed an integrated system consisting of two enrichment stages, (i.e., a deterministic lateral displacement step for depletion of erythrocytes and an immuno-affinity part for leukocyte removal). The putative CTCs were picked up using in-mouth pipette technology followed by single-cell analysis. The authors showed successful detection and isolation of CTCs from 15 of 20 patient samples tested, and consequently conducted single-cell DNA sequencing to show CNVs, SNVs, and **insertion or deletion (InDel).**	[41]
2	Micromanipulation	Surface biomarkers and sub-nanolitre wells used as a guide	Magnetic cell sorting	Prostate cancer	Lohr and colleagues reported an integrated process to isolate, qualify, and sequence whole exomes of isolated single CTCs. They matched 70% mutation similarity of CTCs with the original tissue in prostate cancer patients. In this work, magnetic cell sorting was deployed to enrich CTCs from peripheral blood of patients, that were stained and loaded onto an array of 84 672 sub-nanolitre wells and a micromanipulator was used to transfer the single CTCs to a PCR plate after identification of target cells.	[18]
3	Automated micromanipulation	Size-based selection and surface biomarkers	Parsortix	Breast cancer	Gkountela and coworkers reported a study in which DNA methylation profiles of single CTCs and CTC clusters from 43 breast cancer patients and 13 mouse models were analysed to understand the link between CTC clustering and specific DNA methylation changes that promote stemness and metastasis. The blood samples were enriched for CTCs using the Parsortix system (size-based filtration) prior to transfer to individual PCR tubes using a commercial and automated micromanipulator. A total of 188 single CTCs and 149 CTC clusters were detected and analysed through whole-genome bisulfite sequencing or RNA-sequencing.	[43]
4	Automated micromanipulation	Size-based selection and surface biomarkers	Parsortix	Breast cancer	Reinhardt and colleagues performed single-cell transcriptomic profiling of 33 single CTCs from seven breast cancer patients for characterisation of intercellular heterogeneity in terms of endocrine resistance. They revealed CTC subpopulations with different expression of transcripts regarding the differential phenotypes involved in endocrine signalling pathways and response or resistance to endocrine therapy. In this work authors used the Parsortix system and an automated micromanipulator for isolation and real-time quantitative PCR analysis of individual cells.	[44]
5	LCM	Surface biomarkers	Microfluidic-ratchet (deformability based)	Prostate cancer	Park and colleagues performed single-cell genome sequencing on eight single CTCs using a panel of 73 cancer-related genes. The authors initially enriched the sample for CTCs	[47]

Table 2. (continued)

Study no.	Single-cell isolation technology	Single-cell isolation feature	CTC enrichment technique	Cancer type/organ	Significance and outcomes	Refs
					using a deterministic lateral displacement microfluidic device, followed by a hydrogel encapsulation and LCM to isolate the target cells, showing a 93% single-cell transfer efficiency.	
6	LCM	Surface biomarkers	Immune density	Cancer cell line	Zhu and colleagues demonstrated the potential of carrying out proteomic profiling of five spiked CTCs enriched from whole blood using the immune-density method, followed by single-cell isolation using LCM, nanodroplet sample processing, and ultrasensitive nano-LC-MS. Their workflow could identify an average of 164 protein groups from samples comprising single LNCaP cells (a prostate adenocarcinoma cell line).	[48]
7	FACS	Surface biomarkers	FACS	Breast cancer	Wang and colleagues deployed FACS to separate and isolate single CTCs using a $CD45^-$ and $hTERT^+$ detection scheme. They isolated 11 CTCs from eight breast cancer patients for deciphering SNV profiles and matched 22 co-occurring mutated genes among CTCs and their primary tumours. The authors proposed CTC-shared SNVs as a potential signature for identifying the origin of the primary tumour in a liquid biopsy.	[51]
8	FACS	Surface biomarkers	**Apheresis** followed by immunomagnetic capture via CellSearch	Prostate cancer	Lambros and colleagues used FACS to isolate 185 single CTCs from 14 advanced prostate cancer patients and used whole genome amplification to identify complex inter patient, intercell, genomic heterogeneity missed on bulk biopsy analyses. This was the first study that used the apheresis technique to process large blood volumes (mean volume 59.5 ml) to enrich CTCs in a sample.	[30]
9	Droplet generation	Single-cell RNA sequencing	Size-based filtration	Breast cancer	Brechbuhl and colleagues investigated intravascular interactions between circulating breast cancer cells and other peripheral blood mononuclear cells via single-cell RNA-seq. They predicted enhanced immune evasion in the CTC population with EMT characteristics. They used a commercial and automated single-cell droplet generation package, and 93 CTCs from 11 breast cancer patients were detected throughout their analysis.	[55]
10	Droplet generation	Single-cell RNA-seq	$CD45^-$ enrichment	Hepatocellular carcinoma	D'Avola and coworkers performed single-cell RNA-seq on CTCs from six hepatocellular carcinoma patients in which there is a limited access to tissue samples. They showed that genome-wide expression profiling of CTCs demonstrated CTC heterogeneity, which aids in the detection of known oncogenic drivers in hepatocellular carcinoma such as IGF2. They developed a method that combines image flow cytometry and high density single-cell mRNA sequencing.	[56]

(continued on next page)

Table 2. (continued)

Study no.	Single-cell isolation technology	Single-cell isolation feature	CTC enrichment technique	Cancer type/organ	Significance and outcomes	Refs
11	Droplet generation	Metabolomic activity – lactate production	immunomagnetic WBC depletion	Colorectal cancer	Del Ben and colleagues isolated CTCs through the monitoring of their metabolic activities in droplets. They highlighted a limit of detection as low as ten CTCs among 200 000 WBCs from four patients by leveraging advantage of pH measurement or lactate concentration changes in the extracellular compartment of individual cells without surface antigen labelling.	[59]
12	Droplet generation	Metabolomic activity – lactate production	Immunomagnetic – WBC depletion	Prostate cancer	Rivello and coworkers proposed a metabolic assay chip as a label-free and droplet-based microfluidic device for single-cell extracellular pH measurement for detection and isolation of highly metabolic CTCs. The study was conducted on 56 patients and suggested that the level of metabolic activity of cancer cells can be a prognostic and promising biomarker to study tumour progression and metastasis.	[53]
13	Nano (micro)-wells	Size-based sub-nanolitre wells and molecular analysis (RT-PCR)	Immunomagnetic – $EpCAM^{+}$ selection via MagSifter	NSCLC	Park and colleagues performed single-cell mutation profiling on single CTCs from 55 NSCLC patients, using massively parallel nanowell arrays. First, CTCs were enriched from whole blood samples using MagSifter (using anti-EpCAM antibodies for positive selection), and then the sample was diluted and seeded by direct pipetting and centrifuging on an array of 25 600 wells where cells were isolated individually. Consequently, multigene profiling of individual CTCs was performed through RT-PCR in a high-throughput and multiplexed fashion for single-cell mutation profiling.	[61]
14	Nano (micro)-wells	Size-based sub-nanolitre wells	Immunomagnetic capture via CellSearch	NSCLC	Tamminga and coworkers have shown the potential of using self-seeding nanowells to isolate and assess released CTCs during surgery for NSCLC. The authors isolated over 267 CTCs from ten different NSCLC patients without undergoing surgical resection. Initially, the authors used the CellSearch platform for CTC enrichment from peripheral blood and performed copy number analysis through single-cell whole genome sequencing. The single-cell isolation device operates similarly to a filtration system in which the sample is passed through wells with a 5-μm pore. Larger cells, such as CTCs, clog the wells but allow the remaining sample to flow through the device. Once the well containing the cell of interest is identified, an automated puncher needle approaches and ejects the cell by punching the well and transferring the cell into a collection vessel. The authors concluded that CTCs defined by CellSearch were identified in higher numbers in the pulmonary vein compared with the radial artery and suggested that release of CTCs were not influenced by surgical approach.	[62]

Table 2. (continued)

Study no.	Single-cell isolation technology	Single-cell isolation feature	CTC enrichment technique	Cancer type/organ	Significance and outcomes	Refs
15	Integrated fluidic circuits	Fluidic chambers and single-cell RNA-seq	Size-based inertial microfluidics via ClearCell FX	Breast cancer	Iyer and colleagues used the Polaris system to analyse the transcriptome of 57 single CTCs collected from three different breast cancer patients and compared with 558 single CTC data from publicly available single-cell transcriptome expression profiles of CTCs. They showed CTCs of different cancer types lie on a nearly perfect continuum of EMT values. Additionally, by using full-length transcriptomic analysis they identified several new cell surface biomarkers (ITGB5, TACSTD2, and SLC39A) in addition to the standard EpCAM.	[63]
16	DEP	Surface biomarkers, size and shape and molecular analysis [double-droplet (dd) PCR]	Immunomagnetic – WBC depletion	Melanoma cancer	Tucci and colleagues studied a total of 661 single CTCs from 17 late-stage melanoma patients for the expression of melanoma stem cell markers such as CD271, ABCB5, RANK, and the BRAF mutational status by droplet digital PCR. They used an immunomagnetic negative depletion approach to eliminate CD45-, CD31- or CD34-positive cells, followed by isolation of individual CTCs using a commercial DEPArray machine.	[67]
17	DEP	Single-cell RNA-seq	Parsortix	Renal cell carcinoma	Cappelletti and colleagues studied 21 blood samples from ten patients with metastatic renal cell carcinoma and showed an eightfold amplification of MET in CTCs and a sevenfold increase in cell-free DNA which was correlated with resistance to **crizotinib** and **alectinib**. Authors used Parsortix enrichment technology for enumeration of CTCs, followed by isolation of 37 single CTCs using a DEPArray technology. The isolated CTCs were analysed through next-generation sequencing to identify two subpopulations of epithelial and nonconventional CTCs that lack epithelial and leukocyte markers. DEPArray was also used to isolate CTCs from a patient with stage IV NSCLC who experienced development of resistance to crizotinib and primary resistance to alectinib. Analysis showed a progressive increase in CTC numbers and cell-free DNA during treatment.	[28]

CTCs from enriched sample via fixation/immobilisation of target cells on a slide. Cells are isolated using a highly accurate target recovery and then transferred to a tube or well for various downstream analysis including genomics and transcriptomics analysis. LCM is traditionally labour intensive, time consuming, and requires fixation/immobilisation of samples when dealing with suspended cells [46]. In a study conducted by Park and colleagues, a single-cell sample preparation and genome sequencing analysis was performed on enriched CTCs using hydrogel encapsulation, followed by LCM to isolate the target cells [47]. Furthermore, Zhu and colleagues performed proteomic analysis of spiked CTCs in whole blood using an immune-density method, followed by single-cell isolation using LCM, nanodroplet sample processing, and ultrasensitive nano-liquid chromatography–mass spectrometry (LC–MS) [48].

Box 2. Single-cell analysis types

Captured single CTCs and CTC clusters are primarily analysed at the level of DNA, RNA, proteins, and metabolomics; each providing a unique insight on the state of each cell. It is noteworthy to mention that among the dozens of captured CTCs, only a few are suitable for molecular analysis due to different stress factors including induction of apoptotic factors, immune system attacks, high oxygen levels, and high blood pressure [5,97].

DNA: typical genomic analysis of single CTCs that are of most interest to researchers include SNVs, microsatellite instability, CNVs, large-scale state transitions, and chromosomal rearrangements, which all can provide information on disease stage and behaviour [97].

RNA: studying RNA instead of DNA can provide important insights into active genes in each cell and assist with understanding the complex functionality of CTCs. Single-cell RNA can help with monitoring therapy response, uncover regulatory relationships between genes, and track the trajectories of cell lineages in development [97].

Proteomics: proteomic technology is an essential method used for identification and quantification of protein expression that can distinguish cancer from normal cells due to different protein expression levels [98]. Although analytical platforms and tools for assessing proteomics of single cells (especially CTCs) have lagged behind those for genomics and transcriptomics, multiple studies have suggested dysregulation of specific proteins such as phosphatases and kinases in cancer cells [29,99]. One commercially available proteomics platform is named CyTOF or mass cytometry that has been shown to be able to characterise the protein of a single CTC for studying therapy response, metastasis, immune surveillance, and cell phenotypes [100].

Metabolomics: metabolism is the set of reactions to maintain the living state of cells and includes catabolism (i.e., the conversion of food to energy), anabolism (synthesis of necessary compounds by the cells), and removal of waste. The metabolome is the most representative for predicting a cell phenotype and a good candidate for monitoring cancer cells [101]. Metabolism can provide qualitative and quantitative information on disease state and therapy response for cancer patients and allows for identification of changes in genome, epigenome, and/or proteome, which all can be used as cancer biomarkers and therapy monitoring [102]. However, technical challenges exist for studying metabolites at single-cell resolution due to its small volume and lack of an amplification method [103].

Spatial biology: this is a new frontier in molecular and proteomic biology and refers to the study of tissues within their 2D or 3D environment, down to single-cell resolution. Spatial profiling can help in the understanding of the complex architecture of tissues, revealing vital information on intra- and intercellular heterogeneity, and consequently aid the discovery of the relationship between cell types and defining tissue pathology [104].

Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) is a high-throughput flow cytometry technique that is capable of characterising, detecting, and separating cells via fluorescent tags and allows for sorting of cells by passing an electrostatic charged droplet (containing a cell) through a high-voltage electric field [49]. Most commonly, fluorescently conjugated antibodies are used for measuring and sorting cells based on different protein expression on cell surface. Intracellular detection is also possible but requires fixation and permeabilisation of cells, which compromises some down-stream assays including single-cell RNA-seq [50].

In a study by Wang and colleagues, FACS was deployed to use $CD45^{-}$ and $hTERT^{+}$ markers to isolate CTCs from eight breast cancer patients for measuring **single-nucleotide variants (SNVs)** and matched 22 co-occurring mutated genes among CTCs and their primary tumours [51]. Furthermore, Lambros and colleagues, used FACS to isolate single CTCs from 14 advanced prostate cancer patients and studied them through whole genome amplification and copy-number aberration (CNA) which identified complex inter patient, inter cell, genomic heterogeneity that were missed on bulk biopsy analyses [30].

FACS technologies allow isolation and deposition of nanolitre droplets containing a single cell into a well plate. However, FACS can be limited when dealing with low sample volumes (e.g., enriched CTC samples) due to inherent difficulties including system stabilisation and insufficient sample for cell staining and inability to isolate cells with low expression of target proteins [52].

Droplet generators

Droplet generators leverage the ability of **microfluidics** to precisely handle tiny volumes of liquid (down to picolitres), and are specifically designed to create water-in-oil droplets by mixing these two immiscible fluids (Figure 2) [53,54]. To allow for massively parallel single-cell DNA/RNA analysis, a barcoded bead in lysis buffer is paired with a single cell inside a droplet. Each droplet is used as a reaction chamber where cell lysis occurs, and the DNA/RNA of the cell are tagged with the barcode. In the case of RNA analysis, cDNA is made by reverse transcription and then amplified, followed by pooling all droplets together to construct a library for DNA/RNA sequencing [53]. In a study conducted by Brechbuhl and colleagues, single-cell analysis of CTCs from 11 breast cancer patients were conducted through an initial filtration enrichment followed by single-cell RNA-seq using a commercial and automated droplet-generation package [55]. Similarly, D'Avola and coworkers studied CTCs from six hepatocellular carcinoma patients using a commercial single-cell droplet microfluidic package, indicating the potential of droplet microfluidics for CTC studies for cancer types with limited access to the tissue samples [56].

In addition to high-throughput genomic analysis, droplets can be manipulated by merging, sorting and splitting to test droplet sizes, pH, deformation, and behaviour [57,58]. Droplet-based isolation has allowed a potential application in the study of metabolic activity of CTCs. In line with this, in a study conducted by Del Ben and colleagues, CTCs were isolated inside picolitre droplets and detected via their excessive metabolomic activity (lactate production) and showed potential to detect as little as ten CTCs among 200 000 white blood cells by using pH level measurement of droplets as an alternative to conventional CTC biomarkers [59]. Consequently, Rivello and colleagues further explored this concept and used the pH level measurement of droplets to separate highly metabolomic active cancer cells from blood of cancer patients and conducted a single-cell RNA-seq [53].

Despite their high throughput, droplet generators face difficulties when dealing with low sample input due to system stabilisation times and lowish capture rates and may result in high cell loss. To overcome the droplet instability with low sample input, CTCs can be pooled with background cells. However, the analysis cost per CTC would increase due to inability to select droplets of interest for downstream analysis and there is a risk of cell loss resulting from the inability to completely deconvolute CTCs from mixed pools. In addition, droplet generators have high setup and operational costs, can be complex and require expertise to operate them which may limit the accessibility of these devices.

Nano (micro)-wells

Recently, nanolitre wells have been designed and deployed as a simple method for isolation of single cells. Similar to droplet systems, nanowells are operated by pairing a single cell with a barcoded capture bead for downstream analysis. Cell loading occurs according to a Poisson distribution, and the sample must be diluted to allow the desired single-cell occupancy rate. Both cell and beads are passively loaded through settlement of sample due to gravity, which greatly reduces the need for specialised equipment. Using barcoded beads that are matched to the well size, bead occupancy rates can reach close to 100%. This approach results in many wells that contains no cells, therefore the risk of having wells with multiple cells is lowered, but as each well contains a bead, high cell capture rates are retained [52]. Nanowells are well known as a simple method to analyse single cells for different applications including RNA sequencing [39] and secretion studies [60].

In a study by Park and colleagues, molecular profiling was performed on single CTCs from 55 non-small cell lung cancer patients (NSCLC), using massively parallel nanowell arrays combined

with an on-chip real-time PCR (RT-PCR) [61]. Furthermore, Tamminga and coworkers have shown the potential of using self-seeding nanowells to isolate and assess released CTCs during surgery for NSCLC [62].

Generally, nanowells are simple to operate, low cost, and allow for parallelisation; however, these techniques often suffer from cross-contamination and are not completely suitable for running limited sample including CTCs and other rare cells [39]. It is also worth mentioning that nanowells can be used to enhance the micromanipulation process of single cells through easier detection and retrieval of cells [50].

Integrated fluidic circuits

Integrated fluidic circuits utilise pneumatic membrane valves, pressurised via air, to deflect an elastomer and control fluid movement inside micron-sized channels. In this technique, cells are often encapsulated inside microchambers where multimodal analysis takes place (Figure 2). However, these systems are typically limited in throughput and suffer from high complexity [38]. Iyer and colleagues used the Polaris system (Fluidigm Inc., USA) to analyse the transcriptome of 57 single CTCs collected from three different breast cancer patients and compared them with 558 single CTC data available publicly, and showed an inverse gene expression pattern between PD-L1 and MHC that is implicated in immunotherapy [63].

Dielectrophoresis and optofluidics

Dielectrophoresis (DEP) is a phenomenon that exerts forces on dielectric particles due to a non-uniform electric field. DEP has been deployed to manipulate single cells by utilising electrokinetic principles via combination of microfluidics and microelectronics [64]. Similarly, optofluidic-based isolation approaches combine optics and microfluidics to accurately manipulate particles and cells. These devices provide a high level of control on cell handling, which is effectively used in arrays to isolate single cells and have been shown to be applicable for CTC studies [65,66].

Despite their complexity, DEP and optofluidic arrays have been widely adopted and used to analyse CTCs at single-cell resolution. Tucci and colleagues used DEPArray technology to isolate and analyse CTCs from 17 stage IV cutaneous melanoma patients based on their cell morphology and immunophenotype features, which enabled matching mutational status of CTCs with primary tumours [67]. Furthermore, Cappelletti and colleagues analysed 21 blood samples from ten patients with metastatic renal cell carcinoma using the same DEPArray technology and identified two subpopulations of epithelial and nonconventional CTCs that lacked epithelial and leukocyte markers [28].

Advancements in microfluidic technologies and adoption of DEP and optofluidics for cell handling has led to precise manipulation of single cells for downstream analysis. However, the high complexity and consequently, high cost of these devices just for cell manipulation/isolation is a major drawback for clinical applicability of DEP and optofluidic isolation-based devices.

Current obstacles in single-cell isolation of CTCs

While it is possible to leverage the difference in physical and biological characteristics of CTCs to isolate them from blood, there is currently no single method that would ensure all CTCs from various cancer types are captured within one device. For example, not all CTCs are larger than their noncancerous counterparts and not all CTCs express a cell surface marker that is unique from cells normally found in the blood. This issue is compounded in the case of CTC clusters due to the wider range of cluster size, different morphologies of cells, and the fact that CTC clusters might be composed of non-cancer cells (Figure 4A,B**)**.

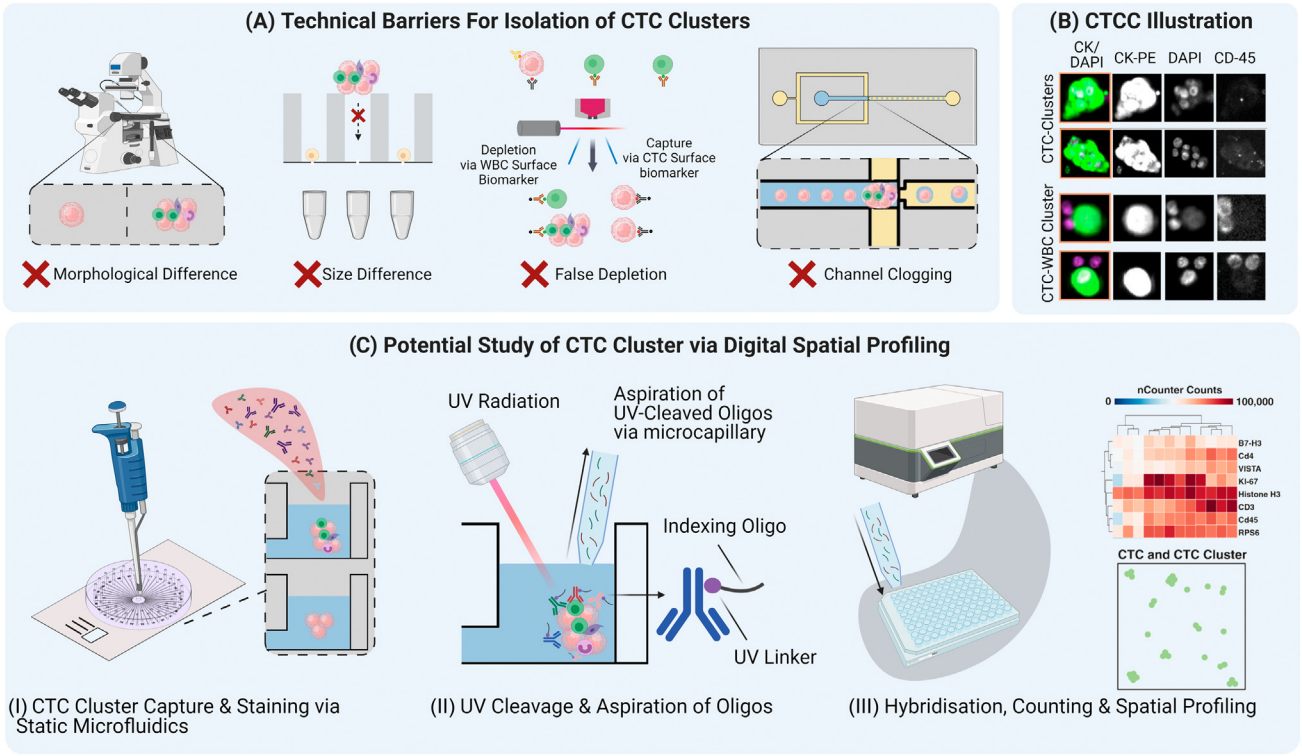

Figure 4. Technical challenges with analysis of circulating tumour cells (CTCs) and potential pathways to study the tumour microenvironment. (A) Technical barriers for isolation of CTC clusters (CTCCs) with the current platforms based on different morphological, size, and surface biomarkers of clusters that may lead to an inability to capture them. (B) Representative images of CTCCs. Adapted from [106] with permission under open license CC BY 4.0. (C) A potential approach for isolating CTC clusters using static microfluidics [27,36] and adopting spatial technologies for efficiently studying them. Created with Biorender. Abbreviation: WBC, white blood cell.

Clinical application of single-cell analysis of single/cluster CTCs in cancer targeted therapies

Personalised cancer therapy aims to treat patients according to individualised genomic profiles in tumours [68]. Studies have reported major resistance mechanisms to targeted therapies across a range of cancer types using genomic analysis of CTCs [9,11]. It is well known that mutations are one of the leading causes of intrinsic and acquired resistance to targeted therapy agents. Considering tumour clonal evolution studies through single-cell analysis, identifying these mutations can be used to monitor tumour evolution trajectories upon therapy pressure and allow the administration of appropriate treatment regimens [69,70]. The molecular characterization of CTCs at the single-cell resolution could help to identify and analyse drug-tolerant clones within the tumour microenvironment (TME), which are clinically defined as a minimal residual disease [71].

To date, most clinical studies evaluating CTC genomic abnormalities have highlighted the presence of gene alterations that can alter the efficacy of target therapies, including but not limited to mutations, rearrangements, or amplifications in *EGFR*, *KRAS*, *HER2*, *PIK3CA*, *ALK*, and *ROS1* [9,11]. For instance, targeting mutated EGFR using an EGFR tyrosine kinase inhibitor (TKI) improved survival rates among patients with NSCLC. Numerous studies have reported mutations that can also be detected in captured CTCs [72,73].

Maheswaran and colleagues isolated CTCs from NSCLC patients identified an in-frame deletion in exon 19, a drug-sensitive-related mutations *EGFR*^{L858R}, and drug-resistance mutation *EGFR*^{T790M} [73]. In agreement with these results, a next-generation-sequencing (NGS)-based analysis of isolated CTCs detected matched *EGFR* mutations between isolated CTCs and the corresponding primary tumour [72]. The presence of genomic rearrangements, particularly in the *ALK* or *ROS1*, have been detected through CTCs analyses [74–76], and a high concordance has been reported for *ALK* rearrangements in CTCs and tumour biopsies in NSCLC [40]. In colorectal cancer (CRC), mutations in codon 12 (G12X) of *KRAS* have been identified in isolated CTCs and positively associated with cancer progression [77]. Since *KRAS*-mutated CTCs can evade EGFR-TKI therapies, continuous monitoring of *KRAS* mutation status using CTCs may facilitate the early detection of developed resistance to EGFR-TKI.

In primary and metastatic breast carcinoma, mutations in *PIK3CA* have been introduced as one of the major molecular resistance mechanisms to HER2-targeted therapy. *PIK3CA* mutations in CTCs have been found in 15.9% of metastatic breast cancer patients [78], with higher rates of *PIK3CA* mutations among CTCs in patients with a HER2-positive status in comparison to HER2-negative status primary tumours [79,80]. Additionally, a positive association between the development of drug resistance and expression of mesenchymal markers in CTCs has been reported in patients with breast and prostate cancer [81,82]. Taken together, these preclinical and clinical findings highlight both the predictive power of genetic alteration analysis of CTCs at the single-cell and the benefit of such analysis in longitudinal studies of those CTCs that display stemness phenotypes during targeted therapy.

Besides gene mutations and rearrangements, CNVs can also be analysed in CTC samples. The analysis of CTCs before the course of treatment can be used to identify distinct CNV signatures in patients with chemosensitive and -resistant small-cell lung cancer (SCLC) and thus highlight molecular mechanisms of disease progression [83]. In contrast to circulating tumour DNA (ctDNA), the detection of mutations and CNVs in CTCs can provide additional information and correlations when it is coupled with specific transcriptomics, proteomics, or morphological analysis [9]. In castration-resistant prostate cancer, gene expression changes to androgen receptor-splice variant seven have been widely investigated in CTCs to explore its role in developing treatment resistance to androgen inhibitors [40,43,84]. Moreover, RNA-seq analysis of single CTCs from patients with resistance to androgen receptor inhibitors also displayed the activation of the glucocorticoid receptor and noncanonical WNT signalling pathways as possible resistance mechanisms [85]. The phenotypic transformation and cellular plasticity are among the main mechanisms of drug resistance across various tumour types, including NSCLC, prostate cancers, and melanoma [70,84,86–88]. Genomic and proteomic analysis of CTCs at the single-cell resolution could provide new insight into the molecular mechanisms behind this phenomenon and aid in identifying appropriate therapies for certain patients experiencing drug resistance. In the neuroendocrine type of prostate cancer, isolated CTCs showed a phenotypic switch associated with endocrine therapy resistance [89]. Additionally, phenotypic transformation to a poorly differentiated phenotype has been observed in CTCs from patients with melanoma who developed relapse in response to BRAF inhibitor (PLX4720) [86].

Interestingly, in the case of breast carcinoma, CTCs from patients with ER$^+$/HER2$^-$ tumour represented a transformation to a HER2$^+$-status-related phenotype under cytotoxic treatment without acquiring additional genetic aberrations [70]. In support of this finding, a recent study demonstrated 73% concordance in ER status and 77% concordance in HER2 status between CTCs and matched primary tumours [90]. To validate these findings in larger cohorts, recently two clinical trials in breast cancer (DETECTIV; NCT02035813) and prostate cancer (CABAV7;

NCT03050866) launched where therapy decisions are based on the cellular and molecular features of CTCs in a personalized manner.

Concluding remarks and future perspectives

CTC enumeration studies have consistently shown a link between CTC numbers and disease outcome. While CTC enumeration has been considered as a powerful prognostic tool, single-cell characterisation technologies that allow deep characterisation of CTCs are now beginning to provide high resolution molecular details about the mechanisms involved with metastasis and therapeutic resistance. These tools are giving us a unique insight into CTC heterogeneity and potentially the primary tumour. The characterisation offered by modern single-cell genomics approaches are providing details of patients' tumour beyond the traditional image-based CTC enumeration. They also supply information in addition to what is covered by ctDNA analysis; namely, which genes are actually being expressed or which mutations are being coexpressed within the same cell. Despite this potential, there are still several technological barriers that must be addressed before CTC and CTC clusters can routinely and accurately be assessed using high dimensional, single-cell molecular assays (see Outstanding questions).

As cancer is a complex disease, often caused by multiple factors involving more than one gene alteration, gaining a true understanding of the clinical relevance of CTCs and CTC clusters across the spectrum of cancer is not a trivial task. It would involve a wide spectrum of studies across many patients with various stages of their disease under different treatment conditions. One approach to expedite the process is to develop high-efficiency isolation approaches that can be coupled to high-resolution molecular profiling tools. Although these assays are becoming increasingly available, they are still prone to biases such as strong stochastic variation, low (and/or uneven) coverage, and high dropout and error rates [91]. Despite the demerits, there is no doubt that genomic analysis can, and has provided a deeper characterisation of CTCs. Clinical studies showed that it might lead to an improved ability for patient stratification for personalised targeted therapies.

An alternative method to further our understanding on how CTCs and CTC clusters relate to cancer progression and treatment selection is to generate CTC cell lines and CTC-derived xenografts (CDX) for molecular analysis and drug screening [91]. *In vitro* results along with *in vivo* validation using CDX enable the identification of anticancer therapeutic agents with increased tumour-killing activity, highlighting the suitability of this approach in principle. However, *in vitro* expansion of the cell line or generation of CDX from CTCs is expected to put selective pressures on the isolated CTCs resulting in potential changes. Additionally, it is impossible to create a platform that allows long-term study of the immune component of the CTC clusters, which plays a critical role in their increased metastatic potential. Therefore, while CTC expansion might be a suitable solution upon the identification of an appropriate growth medium for CTCs, these drug screen assays often require a significant time/cost and CTC expansion may not be performed for every patient.

By combining the results from high-resolution single-cell molecular characterisation of CTCs together with drug screening, it may be possible to connect the phenotypic and genomic profiles of CTCs and CTC clusters to determine inherent drug sensitivities. If drug sensitivity can be strongly linked to the molecular and phenotypic characteristics of the CTCs and CTC clusters, it might be possible to figure out the best treatment for individual patients and to further alter treatments as the disease progresses.

Thus, to enable integration of CTC analysis in clinical settings, enrichment platforms are required to be; (i) simple and cost-effective to operate, (ii) applicable across a wide range of cancers,

Outstanding questions

How can CTC enrichment be improved, in terms of capturing efficiency and output purity, to ease the single cell isolation approach?

How do we lower the costs of single-cell analysis per cell of interest? For instance, most commercial packages and kits do not allow pooling of samples and/or study of small numbers of cells.

Will future CTC enrichment technologies be integrated with single-cell analysis devices in one package to allow use in clinical settings and cause less CTC loss due to sample handling?

Can the current advanced technologies be adopted to efficiently capture and analyse CTC clusters?

In an individual patient with different site of origins (primary tumour, lymph node, or metastatic site), how can CTC analysis at the single-cell level lead to identification of most invasive and therapeutic resistance clones and their genomic alterations?

(iii) allow CTC and CTC clusters to be isolated rapidly prior to any biological changes that are induced, and (iv) highly efficient in capturing viable CTCs and CTC clusters in a format and elution volume that are compatible with current and emerging downstream high dimensional molecular assays. Together, we envision that, technological improvements in CTC isolation, functional profiling of enriched CTCs using state-of-art technologies such as spatial transcriptomic and proteomic profiling, and *ex vivo* expansion of CTCs for drug susceptibility testing are now key to highlight CTC analysis as a potential cancer diagnostic and prognostic biomarker for clinical practice.

Acknowledgements

M. E. W. would like to acknowledge the support of the Australian Research Council through Discovery Project Grants (DP200101860) and the National Health and Medical Research Council through the Career Development Fellowship (APP1143377).

Declaration of interests

N.R. is an employee and stockholder of Fluidigm Corporation. E.S.Y. is an employee and stockholder of NomoCan Corporation. All other authors declare no competing interests.

References

1. Ganesh, K. and Massagué, J. (2021) Targeting metastatic cancer. *Nat. Med.* 27, 34–44
2. Sabnis, A.J. and Bivona, T.G. (2019) Principles of resistance to targeted cancer therapy: lessons from basic and translational cancer biology. *Trends Mol. Med.* 25, 185–197
3. Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646–674
4. Meng, S. *et al.* (2004) Circulating tumor cells in patients with breast cancer dormancy. *Clin. Cancer Res.* 10, 8152–8162
5. Lim, S.B. *et al.* (2019) Single-cell analysis of circulating tumor cells: why heterogeneity matters. *Cancers* 11, 1595
6. Wang, W.-C. *et al.* (2018) Survival mechanisms and influence factors of circulating tumor cells. *Biomed. Res. Int.* 2018, 6304701
7. Kallergi, G. *et al.* (2013) Apoptotic circulating tumor cells in early and metastatic breast cancer patients. *Mol. Cancer Ther.* 12, 1886–1895
8. Khoo, B.L. *et al.* (2016) Single-cell profiling approaches to probing tumor heterogeneity. *Int. J. Cancer* 139, 243–255
9. Cortes-Hernandez, L.E. *et al.* (2020) Molecular and functional characterization of circulating tumor cells: from discovery to clinical application. *Clin. Chem.* 66, 97–104
10. Sun, G. *et al.* (2021) Single-cell RNA sequencing in cancer: applications, advances, and emerging challenges. *Mol. Ther. Oncolytics* 21, 183–206
11. Keller, L. and Pantel, K. (2019) Unravelling tumour heterogeneity by single-cell profiling of circulating tumour cells. *Nat. Rev. Cancer* 19, 553–567
12. Yang, Y.-P. *et al.* (2021) Circulating tumor cells from enumeration to analysis: current challenges and future opportunities. *Cancers* 13, 2723
13. Aktas, B. *et al.* (2009) Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. *Breast Cancer Res.* 11, 1–9
14. Tan, T.Z. *et al.* (2014) Epithelial-mesenchymal transition spectrum quantification and its efficacy in deciphering survival and drug responses of cancer patients. *EMBO Mol. Med.* 6, 1279–1293
15. Gooding, A.J. and Schiemann, W.P. (2020) Epithelial–mesenchymal transition programs and cancer stem cell phenotypes: mediators of breast cancer therapy resistance. *Mol. Cancer Res.* 18, 1257–1270
16. Markiewicz, A. *et al.* (2019) Spectrum of epithelial-mesenchymal transition phenotypes in circulating tumour cells from early breast cancer patients. *Cancers (Basel)* 11, 59
17. Aya-Bonilla, C.A. *et al.* (2017) Isolation and detection of circulating tumour cells from metastatic melanoma patients using a slanted spiral microfluidic device. *Oncotarget* 8, 67355
18. Lohr, J.G. *et al.* (2014) Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. *Nat. Biotechnol.* 32, 479–484
19. Hou, H.W. *et al.* (2013) Isolation and retrieval of circulating tumor cells using centrifugal forces. *Sci. Rep.* 3, 1–8
20. Warkiani, M.E. *et al.* (2014) Slanted spiral microfluidics for the ultra-fast, label-free isolation of circulating tumor cells. *Lab Chip* 14, 128–137
21. Kulasinghe, A. *et al.* (2016) Short term *ex-vivo* expansion of circulating head and neck tumour cells. *Oncotarget* 7, 60101–60109
22. Kulasinghe, A. *et al.* (2017) Circulating tumour cell PD-L1 test for head and neck cancers. *Oral Oncol.* 75, 6–7
23. Kulasinghe, A. *et al.* (2019) The isolation and characterization of circulating tumor cells from head and neck cancer patient blood samples using spiral microfluidic technology. *Methods Mol. Biol.* 2054, 129–136
24. Allard, W.J. *et al.* (2004) Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin. Cancer Res.* 10, 6897–6904
25. Alix-Panabières, C. and Pantel, K. (2014) Challenges in circulating tumour cell research. *Nat. Rev. Cancer* 14, 623–631
26. van der Toom, E.E. *et al.* (2016) Technical challenges in the isolation and analysis of circulating tumor cells. *Oncotarget* 7, 62754–62766
27. Ding, L. *et al.* (2021) An easy-to-operate method for single-cell isolation and retrieval using a microfluidic static droplet array. *Microchim. Acta* 188, 1–11
28. Cappelletti, V. *et al.* (2020) Analysis of single circulating tumor cells in renal cell carcinoma reveals phenotypic heterogeneity and genomic alterations related to progression. *Int. J. Mol. Sci.* 21, 1475
29. Rossi, E. and Rita, Z. (2019) Single-cell analysis of circulating tumor cells: how far we come with omics-era? *Front. Genet.* 10, 958
30. Lambros, M.B. *et al.* (2018) Single-cell analyses of prostate cancer liquid biopsies acquired by apheresis. *Clin. Cancer Res.* 24, 5635–5644
31. Mishima, Y. *et al.* (2017) The mutational landscape of circulating tumor cells in multiple myeloma. *Cell Rep.* 19, 218–224
32. Paoletti, C. *et al.* (2018) Comprehensive mutation and copy number profiling in archived circulating breast cancer tumor cells documents heterogeneous resistance mechanisms. *Cancer Res.* 78, 1110–1122
33. Werner, S. *et al.* (2015) Suppression of early hematogenous dissemination of human breast cancer cells to bone marrow by retinoic Acid-induced 2. *Cancer Discov.* 5, 506–519
34. Nong, J. *et al.* (2018) Circulating tumor DNA analysis depicts subclonal architecture and genomic evolution of small cell lung cancer. *Nat. Commun.* 9, 1–8

35. Powell, A.A. *et al.* (2012) Single cell profiling of circulating tumor cells: transcriptional heterogeneity and diversity from breast cancer cell lines. *PLoS One* 7, e33788
36. Rezaei, M. *et al.* (2021) Simple-to-operate approach for single cell analysis using a hydrophobic surface and nanosized droplets. *Anal. Chem.* 93, 4584–4592
37. Sun, Y.-F. *et al.* (2021) Dissecting spatial heterogeneity and the immune-evasion mechanism of CTCs by single-cell RNA-seq in hepatocellular carcinoma. *Nat. Commun.* 12, 1–14
38. Gross, A. *et al.* (2015) Technologies for single-cell isolation. *Int. J. Mol. Sci.* 16, 16897–16919
39. Gierahn, T.M. *et al.* (2017) Seq-Well: portable, low-cost RNA sequencing of single cells at high throughput. *Nat. Methods* 14, 395–398
40. Antonarakis, E.S. *et al.* (2017) Clinical Significance of Androgen Receptor Splice Variant-7 mRNA detection in circulating tumor cells of men with metastatic castration-resistant prostate cancer treated with first- and second-line abiraterone and enzalutamide. *J. Clin. Oncol.* 35, 2149–2156
41. Xu, M. *et al.* (2020) An integrated microfluidic chip and its clinical application for circulating tumor cell isolation and single-cell analysis. *Cytometry A* 97, 46–53
42. Nelep, C. and Eberhardt, J. (2018) Automated rare single cell picking with the ALS cellcelector™. *Cytometry A J. Int. Soc. Anal. Cytol.* 93, 1267–1270
43. Gkountela, S. *et al.* (2019) Circulating tumor cell clustering shapes DNA methylation to enable metastasis seeding. *Cell* 176, 98–112
44. Reinhardt, F. *et al.* (2019) Diagnostic leukapheresis enables reliable transcriptomic profiling of single circulating tumor cells to characterize inter-cellular heterogeneity in terms of endocrine resistance. *Cancers (Basel)* 11, 903
45. Espina, V. *et al.* (2007) Laser capture microdissection technology. *Expert. Rev. Mol. Diagn.* 7, 647–657
46. Zhang, K. *et al.* (2014) Hand-held and integrated single-cell pipettes. *J. Am. Chem. Soc.* 136, 10858–10861
47. Park, E.S. *et al.* (2018) Isolation and genome sequencing of individual circulating tumor cells using hydrogel encapsulation and laser capture microdissection. *Lab Chip* 18, 1736–1749
48. Zhu, Y. *et al.* (2018) Proteome Profiling of 1 to 5 spiked circulating tumor cells isolated from whole blood using immunodensity enrichment, laser capture microdissection, nanodroplet sample processing, and ultrasensitive nanoLC–MS. *Anal. Chem.* 90, 11756–11759
49. Valihrach, L. *et al.* (2018) Platforms for single-cell collection and analysis. *Int. J. Mol. Sci.* 19, 807
50. Basu, S. *et al.* (2010) Purification of specific cell population by fluorescence activated cell sorting (FACS). *JoVE* 41, e1546
51. Wang, Y. *et al.* (2018) Single nucleotide variant profiles of viable single circulating tumour cells reveal CTC behaviours in breast cancer. *Oncol. Rep.* 39, 2147–2159
52. Nguyen, A. *et al.* (2018) Single cell RNA sequencing of rare immune cell populations. *Front. Immunol.* Published online July 4, 2018. https://doi.org/10.3389/fimmu.2018.01553
53. Matuła, K. *et al.* (2020) Single-cell analysis using droplet microfluidics. *Adv. Biosyst.* 4, 1900188
54. Rakszewska, A. *et al.* (2014) One drop at a time: toward droplet microfluidics as a versatile tool for single-cell analysis. *NPG Asia Mater.* 6, e133
55. Brechbuhl, H.M. *et al.* (2020) Analysis of circulating breast cancer cell heterogeneity and interactions with peripheral blood mononuclear cells. *Mol. Carcinog.* 59, 1129–1139
56. D'Avola, D. *et al.* (2018) High-density single cell mRNA sequencing to characterize circulating tumor cells in hepatocellular carcinoma. *Sci. Rep.* 8, 1–7
57. Teh, S.Y. *et al.* (2008) Droplet microfluidics. *Lab Chip* 8, 198–220
58. Seemann, R. *et al.* (2012) Droplet based microfluidics. *Rep. Prog. Phys.* 75, 016601
59. Del Ben, F. *et al.* (2016) A method for detecting circulating tumor cells based on the measurement of single-cell metabolism in droplet-based microfluidics. *Angew. Chem. Int. Ed.* 55, 8581–8584
60. Zhou, Y. *et al.* (2020) Evaluation of single-cell cytokine secretion and cell-cell interactions with a hierarchical loading microwell chip. *Cell Rep.* 31, 107574
61. Park, S.-M. *et al.* (2016) Molecular profiling of single circulating tumor cells from lung cancer patients. *Proc. Natl. Acad. Sci.* 113, E8379–E8386
62. Tamminga, M. *et al.* (2020) Analysis of released circulating tumor cells during surgery for non-small cell lung cancer. *Clin. Cancer Res.* 26, 1656–1666
63. Iyer, A. *et al.* (2020) Integrative analysis and machine learning based characterization of single circulating tumor cells. *J. Clin. Med.* 9, 1206
64. Di Trapani, M. *et al.* (2018) DEPArray™ system: an automatic image-based sorter for isolation of pure circulating tumor cells. *Cytometry A* 93, 1260–1266
65. Schochter, F. *et al.* (2020) 53BP1 Accumulation in circulating tumor cells identifies chemotherapy-responsive metastatic breast cancer patients. *Cancers* 12, 930
66. Boyer, M. *et al.* (2020) Circulating tumor cell detection and polyomavirus status in Merkel cell carcinoma. *Sci. Rep.* 10, 1–13
67. Tucci, M. *et al.* (2020) Dual-procedural separation of CTCs in cutaneous melanoma provides useful information for both molecular diagnosis and prognosis. *Ther. Adv. Med. Oncol.* 12, 1758835920905415
68. Aboulkheyr Es, H. *et al.* (2018) Personalized cancer medicine: an organoid approach. *Trends Biotechnol.* 36, 358–371
69. Paolillo, C. *et al.* (2017) Detection of activating estrogen receptor gene (ESR1) mutations in single circulating tumor cells. *Clin. Cancer Res.* 23, 6086–6093
70. Jordan, N.V. *et al.* (2016) HER2 expression identifies dynamic functional states within circulating breast cancer cells. *Nature* 537, 102–106
71. Dagogo-Jack, I. and Shaw, A.T. (2018) Tumour heterogeneity and resistance to cancer therapies. *Nat. Rev. Clin. Oncol.* 15, 81–94
72. Marchetti, A. *et al.* (2014) Assessment of EGFR mutations in circulating tumor cell preparations from NSCLC patients by next generation sequencing: toward a real-time liquid biopsy for treatment. *PLoS One* 9, e103883
73. Maheswaran, S. *et al.* (2008) Detection of mutations in EGFR in circulating lung-cancer cells. *N. Engl. J. Med.* 359, 366–377
74. Pailler, E. *et al.* (2015) High level of chromosomal instability in circulating tumor cells of ROS1-rearranged non-small-cell lung cancer. *Ann. Oncol.* 26, 1408–1415
75. Pailler, E. *et al.* (2016) Method for semi-automated microscopy of filtration-enriched circulating tumor cells. *BMC Cancer* 16, 1–15
76. Pailler, E. *et al.* (2017) Circulating tumor cells with aberrant ALK copy number predict progression-free survival during crizotinib treatment in ALK-rearranged non-small cell lung cancer patients. *Cancer Res.* 77, 2222–2230
77. Liu, Y. *et al.* (2017) Meta-analysis of the mutational status of circulation tumor cells and paired primary tumor tissues from colorectal cancer patients. *Oncotarget* 8, 77928–77941
78. Schneck, H. *et al.* (2013) Analysing the mutational status of PIK3CA in circulating tumor cells from metastatic breast cancer patients. *Mol. Oncol.* 7, 976–986
79. Gasch, C. *et al.* (2016) Frequent detection of PIK3CA mutations in single circulating tumor cells of patients suffering from HER2-negative metastatic breast cancer. *Mol. Oncol.* 10, 1330–1343
80. Pestrin, M. *et al.* (2015) Heterogeneity of PIK3CA mutational status at the single cell level in circulating tumor cells from metastatic breast cancer patients. *Mol. Oncol.* 9, 749–757
81. Markou, A. *et al.* (2018) Multiplex gene expression profiling of *in vivo* isolated circulating tumor cells in high-risk prostate cancer patients. *Clin. Chem.* 64, 297–306
82. Yu, M. *et al.* (2013) Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science* 339, 580–584
83. Carter, L. *et al.* (2017) Molecular analysis of circulating tumor cells identifies distinct copy-number profiles in patients with chemosensitive and chemorefractory small-cell lung cancer. *Nat. Med.* 23, 114–119
84. Jolly, M.K. *et al.* (2018) Phenotypic plasticity, bet-hedging, and androgen independence in prostate cancer: role of non-genetic heterogeneity. *Front. Oncol.* 8, 50
85. Miyamoto, D.T. *et al.* (2015) RNA-Seq of single prostate CTCs implicates noncanonical Wnt signaling in antiandrogen resistance. *Science* 349, 1351–1356

86. Tsao, S.C. *et al.* (2018) Characterising the phenotypic evolution of circulating tumour cells during treatment. *Nat. Commun.* 9, 1–10
87. Chen, P.Y. *et al.* (2018) Adaptive and reversible resistance to Kras inhibition in pancreatic cancer cells. *Cancer Res.* 78, 985–1002
88. Oser, M.G. *et al.* (2015) Transformation from non-small-cell lung cancer to small-cell lung cancer: molecular drivers and cells of origin. *Lancet Oncol.* 16, e165–e172
89. Beltran, H. *et al.* (2016) The initial detection and partial characterization of circulating tumor cells in neuroendocrine prostate cancer. *Clin. Cancer Res.* 22, 1510–1519
90. Magbanua, M.J.M. *et al.* (2018) Expanded genomic profiling of circulating tumor cells in metastatic breast cancer patients to assess biomarker status and biology over time (CALGB 40502 and CALGB 40503, Alliance). *Clin. Cancer Res.* 24, 1486–1499
91. Diamantopoulou, Z. *et al.* (2020) Circulating tumor cells: ready for translation? *J. Exp. Med.* 217
92. Chen, X.-X. and Bai, F. (2015) Single-cell analyses of circulating tumor cells. *Cancer Biol. Med.* 12, 184
93. Yu, M. *et al.* (2014) *Ex vivo* culture of circulating breast tumor cells for individualized testing of drug susceptibility. *Science* 345, 216–220
94. Andree, K.C. *et al.* (2016) Challenges in circulating tumor cell detection by the CellSearch system. *Mol. Oncol.* 10, 395–407
95. Khoo, B.L. *et al.* (2017) Advancing techniques and insights in circulating tumor cell (CTC) research. In *Ex Vivo Engineering of the Tumor Microenvironment* (Aref, A.R. and Barbie, D., eds), pp. 71–94, Springer International Publishing
96. Ferreira, M.M. *et al.* (2016) Circulating tumor cell technologies. *Mol. Oncol.* 10, 374–394
97. Hwang, B. *et al.* (2018) Single-cell RNA sequencing technologies and bioinformatics pipelines. *Exp. Mol. Med.* 50, 1–14
98. Hanash, S. and Taguchi, A. (2011) Application of proteomics to cancer early detection. *Cancer J. (Sudbury, Mass.)* 17, 423
99. Nusinow, D.P. *et al.* (2020) Quantitative proteomics of the cancer cell line encyclopedia. *Cell* 180, 387–402
100. Spitzer, M.H. and Nolan, G.P. (2016) Mass cytometry: single cells, many features. *Cell* 165, 780–791
101. Armitage, E.G. and Barbas, C. (2014) Metabolomics in cancer biomarker discovery: current trends and future perspectives. *J. Pharm. Biomed. Anal.* 87, 1–11
102. Johnson, C.H. *et al.* (2016) Metabolomics: beyond biomarkers and towards mechanisms. *Nat. Rev. Mol. Cell Biol.* 17, 451–459
103. Duncan, K.D. *et al.* (2018) Quantitative mass spectrometry imaging of prostaglandins as silver ion adducts with nanospray desorption electrospray ionization. *Anal. Chem.* 90, 7246–7252
104. Merritt, C.R. *et al.* (2020) Multiplex digital spatial profiling of proteins and RNA in fixed tissue. *Nat. Biotechnol.* 38, 586–599
105. Smietanka, U. *et al.* (2021) Clusters, assemblies and aggregates of tumor cells in the blood of breast cancer patients; composition, mode of action, detection and impact on metastasis and survival. *Int. J. Transl. Med.* 1, 55–68
106. Costa, C. *et al.* (2020) Analysis of a real-world cohort of metastatic breast cancer patients shows circulating tumor cell clusters (CTC-clusters) as predictors of patient outcomes. *Cancers* 12, 1111