Tumor Profiling

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Beyond the Capture of Circulating Tumor Cells: Next-Generation Devices and Materials

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Over the last decade, significant progress has been made towards the development of approaches that enable the capture of rare circulating tumor cells (CTCs) from the blood of cancer patients, a critical capability for noninvasive tumor profiling. These advances have leveraged new insights in materials chemistry and microfluidics and allowed the capture and enumeration of CTCs with unprecedented sensitivity. However, it has become increasingly clear that simply capturing and counting tumor cells launched into the bloodstream may not provide the information needed to advance our understanding of the biology of these rare cells, or to allow us to better exploit them in medicine. A variety of advances have now emerged demonstrating that more information can be extracted from CTCs with next-generation devices and materials featuring tailored physical and chemical properties. In this Minireview, the last ten years of work in this area will be discussed, with an emphasis on the groundbreaking work of the last five years, during which the focus has moved beyond the simple capture of CTCs and gravitated towards approaches that enable in-depth analysis.

1. Introduction

The progression of cancer from a localized to metastatic disease is central to the often devastating effects of this complex illness.[1–4] The formation of metastases in organs distant from a primary tumor is proposed to occur because of the release of circulating tumor cells (CTCs) into the bloodstream (Figure 1).[5–8] If CTCs possess an aggressive phenotype and are able to invade a tissue, secondary tumors are formed, which are often deadly. It is therefore essential to understand how the presence and properties of CTCs impact the progression of cancer.

The capture and analysis of CTCs is very challenging owing to the low levels of these cells in blood.[9,10] A single CTC in a milliliter of blood can be clinically relevant, and in this same volume, billions of red blood cells (RBCs) and millions of white blood cells (WBCs) are also present. Hence, effective capture requires a high level of specificity for CTCs and the ability to handle very low cell numbers. Furthermore, several milliliters of blood must be processed, so throughput must be high.

The first approaches developed for CTC analysis included immunocytology,[11] flow cytometry,[12] and magnetic separation.[13] It was the latter technique, combined with immunofluorescence, that provided the basis for the first FDA-cleared instrument for CTC analysis, CellSearch. Developed in 1999, CellSearch is an immunomagnetic enrichment method that relies on targeting a marker specific to epithelial cells, the epithelial cell adhesion molecule (EpCAM). CellSearch represents the most widely used technique in the clinical setting and is still the only CTC detection method with FDA clearance.

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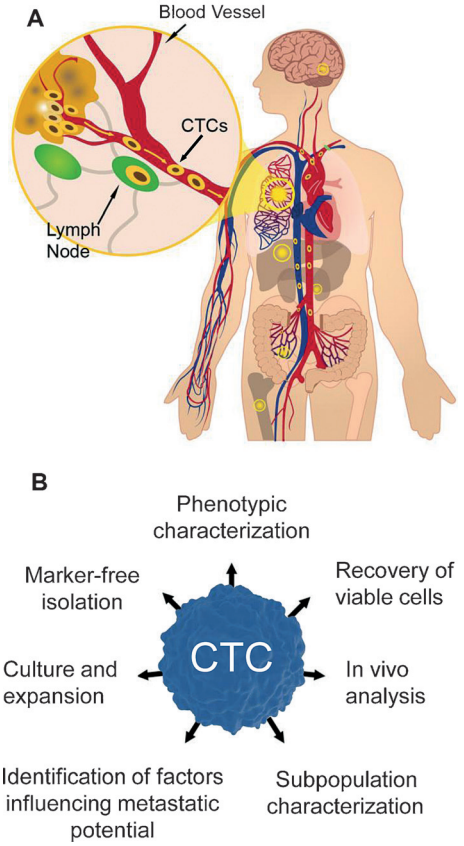

Figure 1. Circulating tumor cells (CTCs). A) Circulating tumor cells are released from the primary tumor and enter the bloodstream, where they invade secondary sites and form metastatic tumors. Image adapted from Ref. [2]. B) Emerging areas of research for enabling new types of CTC analysis; these areas are summarized in this Minireview.

The CellSearch approach labels CTCs with magnetic particles coated with anti-EpCAM antibodies, captures the cells from whole blood, and automates their imaging. CTCs are defined as cells that display a DAPI-stained nucleus and co-express EpCAM and cytokeratins while not expressing the pan-leukocyte marker CD45.[6] Numerous clinical studies of CTC levels have been conducted using the CellSearch system[14–20] and have demonstrated that monitoring these cells can provide powerful prognostic information for a subset of cancers.

While CellSearch has allowed more thorough studies of the clinical relevance of CTCs, it has a number of limitations. Several studies have indicated that this approach has an inherent lack of sensitivity that limits its applicability to the analysis of cells with high EpCAM levels.[21,22] An additional constraint is the inability to access cellular material after cells are enumerated.

This Minireview will describe the progress that has been made in the last decade to advance systems addressing these limitations. Table 1 summarizes a series of breakthroughs that will be discussed. Remarkable improvements in the sensitivity, specificity, and versatility of CTC capture and analysis systems have been realized that allow CTCs to be measured with unprecedented precision. An important new direction in this field is the development of devices and materials that provide information beyond CTC enumeration. New approaches for capture that integrate multiple recognition handles, or eliminate the need for predetermined capture agents are allowing more diverse collections of CTCs to be isolated. Integrated devices that allow separation of heterogeneous CTCs or enable phenotypic and molecular profiling are facilitating more in-depth characterization of these cells. Progress has also been made in the development of materials that facilitate the culture and expansion of CTCs, a very

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important capability for the development of personalized treatment plans. These breakthroughs will affect the study of these rare cells and their eventual use in the clinical management of cancer.

2. Engineering Effective CTC Capture Devices and Materials

The tantalizing possibility that CTCs could serve as a liquid biopsy, revealing the molecular-level signature of a tumor, has prompted intensive effort focused on the development of high-performance CTC capture methods. In order to provide greater levels of sensitivity than are achievable with CellSearch, a variety of techniques have been explored to isolate these cancer cells from the blood. Comprehensive reviews of work in this area have described the diverse systems now available for CTC isolation.[23–26] To set the stage for the new directions in CTC analysis, we will briefly describe the several high-performance methods developed in the last decade in the following section.

2.1. Affinity-Based Isolation of CTCs

Next-generation affinity capture approaches offer significant improvements in the sensitivity and specificity of CTC capture and analysis. Antibody-modified microdevices and nanomaterials have enhanced the capture efficiencies of CTCs from patient samples and allowed detailed molecular-level characterization of CTCs to be performed. While EpCAM remains the predominant capture target for affinity-based approaches, antibodies are interchangeable as capture agents, which broadens the applicability of these systems to non-epithelial tumors or low EpCAM CTCs.

One of the first microfluidic CTC affinity capture systems to be reported demonstrated the remarkable gains in performance that could be achieved with a microscale approach.[27] This device featured microposts etched into a silicon substrate that were functionalized with anti-EpCAM. The microposts were positioned to promote maximal contact with the cells flowing through the device (Figure 2A). After blood processing, immunostaining was used to identify CTCs that were positive for cytokeratin and negative for CD45. High capture efficiencies were attained with a variety of cell lines, and detectable levels of CTCs were observed in 115 out of 116 patient samples analyzed. This system was the first that could process whole blood directly, and it was proposed that the lack of preprocessing steps was a factor in increasing the sensitivity of CTC detection. Promoting interactions between CTCs and an antibody-modified surface by using microfluidic flow also likely played a role in the improved performance.

Many other affinity-based microfluidic capture systems followed this groundbreaking work.[28,29] Devices featuring micropatterned surfaces that promote turbulence and high levels of collisions between CTCs and immobilized antibodies have been engineered,[30] as well as integrated systems with electrical detectors for CTC counting.[31] In addition, the use of microfluidic sorting systems that can separate CTCs labeled with fluorescent antibodies through partitioning into nanoliter aliquots enabled the isolation of these cells without any need for detachment from the device.[32]

Progress in developing devices that permit the recovery of CTCs after capture has also been made. In particular, the MagSweeper, a rod-like device that can collect CTCs from clinical samples, has provided a solution for the isolation of patient CTCs for detailed characterization (Figure 2B).[33–35]

Nanomaterials have been shown to further enhance the sensitivity of CTC capture. A NanoVelcro chip, based on an array of nanoscale silicon needles functionalized with antibodies against CTC surface markers, has been shown to provide an optimal environment to promote the adhesion of CTCs to the capture substrate (Figure 2C).[36–38] Other nanomaterials, such as conducting polymer nanodots[39] and electrospun TiO_2 nanofibers,[40] have been tested and optimized for CTC capture, and the optimal nanoscale roughness for efficient cell binding has been estimated.

2.2. Size-Based Separation of CTCs

One of the first approaches to arise as an alternative to the immunomagnetic capture of CTCs leveraged the difference in size that exists between tumor cells and the normal cell types found in the blood. Some tumor cells possess diameters that are larger than normal RBCs and WBCs, thus presenting the possibility that they could be isolated with high-precision filtration. Early work with sieve-like materials indicated that cancer cells could be separated from the blood,[41] and soon after, microfabricated filters were produced with more-precisely engineered features (Figure 2D,E).[42]

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Table 1: Summary of CTC capture and analysis approaches.

CTC capture/ analysis technology	Description	Advantages/Limitations	Ref.
CellSearch	FDA-cleared Immunomagnetic enrichment method	Limited to capture of CTCs with high EpCAM levels	[14–20]
MagSweeper	Immunomagnetic enrichment	One of the first non-destructive capture methods	[34]
Nanovelcro	Nanovelcro surface with high surface-to-volume ratio enables capture of single tumor cells	Stability of antibody-functionalized chip is critical	[92]
ISET[a]	Marker-free filtration approach	Limited selectivity due to the inherent size overlap between CTCs and WBCs	[44, 46]
Flow fractionation combined with dielectrophoresis	Separation technique based on total cell capacitance	Cytoplasmic and membrane conductivities are likely to change over the course of CTC capture	[51]
CTC iChip	Hydrodynamic sorting, inertial focusing, and magnetophoresis CTC capture	Negative depletion option allows marker-free isolation of CTCs	[62]
Gelatin nanocoatings	Antibody-functionalized coating enables thermal or mechanosensitive release of captured CTCs	Gentle release mechansism	[69]
Aptamer-modified silicon nanowires	Aptamer-based capture of CTCs	Mild release conditions (nucleases or anti-sense DNA strands) allow recovery of viable cells. Stability of aptamers is compromised in blood	[67]
EpCAM-functionalized graphene oxide	Capture and culture of CTCs on graphene oxide surface	Cells can be cultured directly within device	[72]
CTC subpopulation sorting chip	Device that sorts CTC subpopulations with differing surface expression profiles into discrete microfluidic zones	Allows CTC heterogeneity to be profiled	[83]
Cluster-Chip	CTC clusters (2–30 cells) are captured using bifurcating traps under low shear stress conditions.	Label-free approach allows gentle release	[88]
Integrated capture and electrochemical detection chip	Integrated circuit for CTC capture, lysis, and mRNA analysis with processing times within 1 h	Integration of capture and analysis limits sample handling and associated artifacts	[98]
μHall chip	μHall detection allows simultaneous analysis of multiple markers labeled with different magnetic nanoparticles	Multi-marker profiles collected. Detection may not be suitable for cells with extremely weak biomarkers, such as stem cells, due to low magnetic susceptibility	[99, 100]
Nanoflares	Nanoparticle fluorescent probes that enter cells, and bind to a target mRNA sequence. After binding, a fluorophore is released.	The first gene-based approach for characterizing live cancer cells from blood. Flow-cytometry-dependent analysis may require larger populations of cells	[101]
Multiwell invasion chip (MI-Chip)	Device that measures 3D cancer-cell migration towards a chemotactic agent	Phenotypic information reported. Assays demonstrated for cell lines, not CTCs	[103]
In vivo CTC detector	EpCAM- functionalized stainless steel medical Seldinger guidewire inserted intravenously for detection of CTCs	In vivo analysis provides real-time monitoring of CTCs. CTCs do not undergo damage that can occur during typical blood processing	[108]

[a] ISET = Isolation by size of epithelial tumor cells.

Polycarbonate membranes, which can be etched with an ion beam to produce arrays of consistently-sized pores, provided an ideal platform to optimize the performance of size-based CTC isolation.[43–45] A technique referred to as isolation by size of epithelial tumor cells (ISET)[44,46] was developed by using this material platform, and this technique enabled very sensitive enumeration of CTCs in patient samples. Furthermore, it was shown to be compatible with gene-based analysis of the CTCs, either through polymerase chain reaction (PCR) or fluorescence in situ hybridization

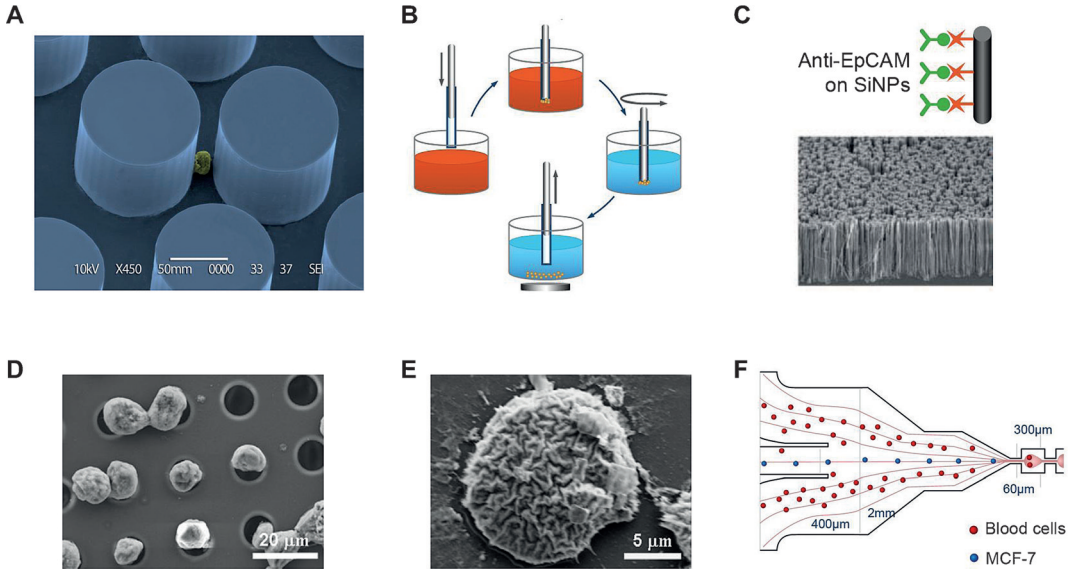

Figure 2. Advanced CTC isolation techniques with improved sensitivity. A) Scanning electron microscopy (SEM) image of a single non-small-cell lung cancer cell captured on the side of an antibody-functionalized micropost.[27] B) MagSweeper, a rod-like device used to collect magnetically tagged CTCs from clinical samples.[33] C) Antibody-coated silicon nanopillars (SiNPs) that promote interactions between CTCs and a substrate.[37] D, E) SEM images showing prostate cancer cells captured on a porous parylene membrane filter.[42] F) A microfluidic device that uses multi-orifice flow fractionation (MOFF) and dielectrophoresis (DEP) to capture circulating tumor cells. This system enables continuous, high-throughput separation of CTCs from white blood cells through the application of external DEP forces.[51] Figures adapted or reprinted with permission from Refs. [27, 33, 37, 42, 51].

(FISH). This approach was subsequently extended to a wider size range of CTCs by the inclusion of beads that would specifically bind to the cells and increase their diameters, although this does introduce dependence on cell surface markers.[47] Photodetachment of the beads after capture was used to ensure minimal interference with cell imaging.

Other size-based isolation methods have harnessed microfluidic separation rather than filtration.[48,49] A platform using inertial focusing and trapping of CTCs in microscale vortices has achieved good levels of sensitivity and specificity, with purity values close to 95% and moderate capture efficiency. Turnaround times are fast, with a 7.5 milliliter sample taking only 20 min to process. Therefore, this method provides a rapid means to isolate high-purity samples of CTCs.

Size-selection systems are unique in that they offer label-free isolation of viable CTCs for post-capture analysis. However, this approach cannot achieve perfect separation, since there is a proportion of CTCs that are smaller or the same size as WBCs.[47] This distinction can reduce the purity of the enriched population.

2.3. Dielectric Separation of CTCs

Cancer cells can also be separated from blood by using dielectrophoresis (DEP).[50,51] This label-free approach exploits the intrinsic dielectric differences between cell types. Cell dielectric measurements report the total plasma membrane capacitance and conductance by using alternating field gradients. Total capacitance can be used to separate tumor cells from white blood cells.

CTCs can be isolated by using dielectrophoresis field-flow fractionation (depFFF), where DEP forces are combined with drag flow to fractionate a sample of different types of particles. After capture, viable tumor cells can be recovered for culture or molecular analysis. DepFFF is able to recover up to 90% of the tumor cells spiked into blood.[50,51] Tumor cells are depleted from the blood when the applied DEP frequency is 60 kHz, and the cells are concentrated in fractions following a frequency shift to 15 kHz.

Multi-orifice flow fractionation (MOFF) has been combined with DEP to isolate breast cancer cells from blood. Hydrodynamic sorting based on inertial microfluidics is achieved by using multi-orifice structures (Figure 2F).[51] The combination of hydrodynamic sorting with DEP results in extremely high separation efficiencies.

Another application of DEP induces dielectrophoretic forces through the application of light, and is used to isolate CTCs in a laminar-flow microfluidic environment.[52] Cancer cells are separated from white blood cells with a purity of up to 82%.

Limitations to these methods arise because cytoplasmic and membrane conductivities may change over the course of the separation process. Separation periods can be optimized to minimize these variations and reduce cell–cell interactions.

3. Beyond Capture and Enumeration: Next-Generation Devices and Materials

The methods described in the previous section provided the means to isolate and study CTCs with improved sensitivity and efficiency, and helped to demonstrate that it might be

possible to one day turn a blood sample into a liquid biopsy that would report on the molecular-level properties of a tumor. Over the last several years however, it has become increasingly apparent that new capabilities are needed to make this possibility a reality. Recent studies have shown that a given patient may have many different types of CTCs in their blood, and that only a subset may possess a metastatic phenotype.[53–55] Furthermore, the heterogeneity of CTCs necessitates that the capture is performed by using a variety of markers, or by using marker-free approaches that can isolate phenotypically diverse cancer cells. Recovering and culturing CTCs after capture so that they can be further analyzed for drug susceptibility and metastatic potential is critical.[56] Developing devices that can not only isolate CTCs but also characterize their molecular properties in situ are sought to better characterize these cells. Furthermore, the in vivo analysis of CTCs is an important goal.

3.1. Marker-Free Isolation of CTCs

A holy grail in CTC isolation is to retrieve these cells without the requirement for a specific marker. While new technologies have incorporated multiple markers[53,57,58] and leveraged multivalency[58–60] for highly specific capture, marker-free CTC isolation would enable CTCs with uncharacterized phenotypes to be analyzed. Marker-free isolation presents a means to avoid the selection bias that results from targeting specific surface markers for capture.

A recent breakthrough in marker-free isolation of CTCs was enabled by a negative depletion strategy and an inertial focusing chip referred to as the iChip.[61,62] An initial hydrodynamic sorting chamber separates RBCs and platelets from CTCs and WBCs on the basis of size (Figure 3). Then, the sample is incubated with antibody-tagged magnetic particles that target either WBCs or CTCs, and the cells are then separated into a bulk fraction. If WBCs are captured with magnetic particles, this allows the CTCs to be isolated without the use of a specific marker for their identification. This approach was applied to the isolation of CTCs in patients with triple-negative breast cancer, which represents a challenge for EpCAM-based capture because the CTCs primarily express mesenchymal markers. In addition, the negative depletion iChip was extended to other challenging CTC types, including those derived from melanoma and pancreatic cancer patients.

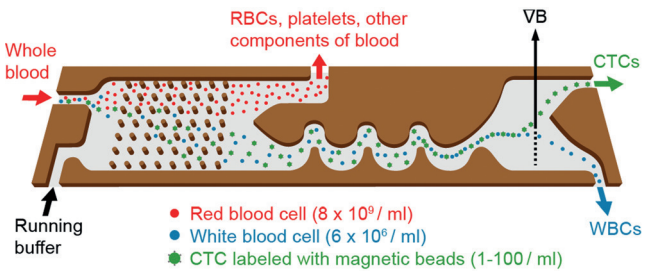

Figure 3. The CTC-iChip, which is composed of different regions that facilitate size-based deflection, inertial focusing, and magnetophoresis to separate WBCs from CTCs.[62] Image adapted from Ref. [62].

A drawback of this method is the relatively higher level of contaminating WBCs compared to what is typically obtained with positive selection methods.

Marker-free approaches for CTC isolation are powerful tools that will enable heterogeneous types of cancer cells to be studied and may play a significant role in determining which cells possess an invasive phenotype. Increasing the stringency of CTC purification achieved with this type of device represents a principle goal that will facilitate the characterization of more diverse CTCs without interference from healthy blood cells.

3.2. Capture and Release of CTCs

The first methods developed for CTC analysis were designed to facilitate the identification of these cells via immunostaining, but the use of destructive characterization was quickly recognized as a constraint that would limit downstream analysis of the genetics and proteomics of these cells. Releasing viable cells allows for further analysis such as quantitative PCR, whole genome sequencing, and xenograft studies,[54,63,64] which are essential for fully understanding cancer metastases. This has prompted a search for gentle conditions that could be used to release fragile CTCs from capture devices. Over the last several years, a variety of systems have permitted the efficient recovery of cancer cells after capture by using chemical,[65] enzymatic,[66,67] self-assembly,[68] mechanosensitive,[69] and thermal release[70,71] mechanisms (Figure 4). High levels of cellular viability have been achieved for cancer cells isolated with low levels of contaminating cells. This is a new capability that will enhance our understanding of the biological properties of CTCs and their medical relevance.

Recovering viable cancer cells after antibody-based capture is a challenge because of the high affinity of antibodies to their respective surface antigens. Digestion of cell-surface proteins has been pursued as a means to unlink antibody/antigen complexes, but low recovery efficiencies were obtained.[31] Recent work on alternative methods has included the use of labile metal ion linkers between nanoparticles and antibodies that can be displaced with EDTA,[65] and gelatin-based nanocoatings that can be denatured upon heating above 30 °C.[69] The latter approach can also be used to release single CTCs with mechanical force. Another thermoresponsive technique relies on the use of immobilized polymer brushes that internalize the attached antibodies at low temperatures,[70,71] an effect that can be used to release CTCs upon cooling of a brush-modified substrate. This approach permitted the isolation of CTCs from patient samples and sequencing of tumor-related mutations.

Using aptamers instead of antibodies as capture agents presents an alternative capture approach that is conducive to a variety of options for the release of viable cells. Aptamers immobilized within large DNA networks[66] or on silicon nanowires (SiNWs)[67] have been used for cell capture and then treated with nucleases to allow the cells to be recovered. Aptamer-modified SiNWs achieved a capture efficiency of 95% and a recovery rate of 94% for lung cancer cells.[67]

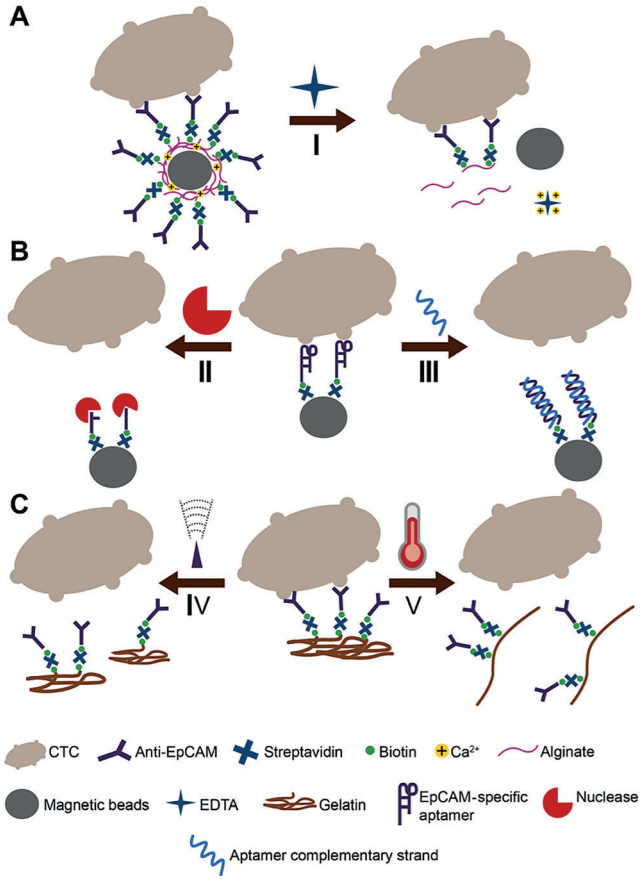

Figure 4. Mechanisms of CTC release. A) Chemical release of CTCs. Magnetic beads are coated with alginate through layer-by-layer deposition in the presence of Ca^{+2}, and EpCAM specific antibodies are attached to the alginate coat through biotin/streptavidin binding. The addition of EDTA (I) leads to sequestration of the Ca^{+2} ions, thereby disrupting the interaction between the cells and the coated beads and subsequently causing cell release.[65] B) Enzymatic- and self-assembly-based release of CTCs. CTCs are captured using aptamer-tagged magnetic beads. Cells are separated from the beads using either nucleases (II), which digest the aptamers, or a sequence complementary to the aptamer (III), which forces the cells to dissociate from the cell/aptamer complex.[66–68] C) Mechanosensitive and thermal release of CTCs. CTCs are captured on an antibody-functionalized gelatin surface. Cells are released by either applying a mechanical force (IV) or denaturing the gelatin nanocoating by increasing the temperature above 30 °C (V).[69]

Alternatively, a nucleic acid with a sequence complementary to that of the capture aptamer can be used to trigger cell release.[68] Aptamer-based methods therefore allow cell release under mild conditions that appear to facilitate the recovery of viable cells, but these methods have not been tested in blood, potentially because of aptamer degradation by natural nucleases.

3.3. Enabling Culture and Expansion of CTCs

The isolation and recovery of CTCs permits the application of single-cell methods that can extract powerful information from these cells. However, the specific factors that drive CTCs to form secondary tumors remain unidentified, which makes culture assays essential for characterization. Phenotypic analysis of metastatic potential or chemosensitivity requires that viable cells be kept alive and cultured, which requires an environment that promotes cell growth and division. Establishing cultures from primary CTCs has enabled patient drug sensitivity to be monitored,[56] and may allow subpopulations of CTCs[54,55] to be better characterized for metastatic potential.

A number of devices and materials show promise for the culture of CTCs. A recent study of a graphene oxide based immunoaffinity capture device[72] showed that cancer cells could be cultured directly on the oxide surface after capture. Moreover, the use of magnetic nanoparticles[73] for cell capture has also allowed the expansion of viable cells. Microfluidic devices with embedded microstructures for immunocapture[74] or compartments for trapping cells with antibody-targeted magnetic nanoparticles attached[75] are also effective for post-capture culture. The latter device was also used to monitor CTCs in a mammary-tumor bearing mouse model, a capability that only few devices exhibit because of the very small samples that can be obtained from this type of animal model.

3.4. Identification of CTC Subpopulations and Visualizing Heterogeneity

Tumors are intrinsically heterogeneous, with cells that possess divergent phenotypes according to exposure to different microenvironments and therapeutics.[76,77] Cells that detach from a tumor and enter the bloodstream may continue to evolve different properties as they persist in the bloodstream, and several studies have elucidated heterogeneous transcriptional levels and surface expression in CTCs.[34,78] The epithelial-to-mesenchymal transition (EMT) is one set of cellular changes that appears to occur as tumor cells evolve away from their epithelial origins and acquire the greater plasticity needed for metastasis.[79] EMT is therefore a source of dynamic heterogeneity in CTCs. The identification of specific subpopulations of CTCs with pronounced metastatic potential further illustrates the fact that these cells should not be considered to be phenotypically identical to their counterparts within a solid tumor (Figure 5A).[80] Morphological heterogeneity can also be indicative of metastatic potential owing to changes in prometastatic cell signaling pathways. Recently, very high levels of small nuclear CTCs were shown to be elevated in prostate cancer patients with visceral metastatic disease.[81]

Sources of dynamic and static heterogeneity present a challenge for CTC capture and characterization. Microfabricated devices engineered to disrupt cell–cell interactions have been used to study cultured cells as they diverge into different phases of EMT,[82] and have shown that cells in different phases of this transition exhibit differing levels of susceptibility to chemotherapeutics, but this approach remains untested on patient CTCs. Fluorescence-activated cell sorting (FACS) has been used to sort CTC subpopulations in patient samples.[54,55] However, it is not effective with all subpopulations and requires large samples of blood that are

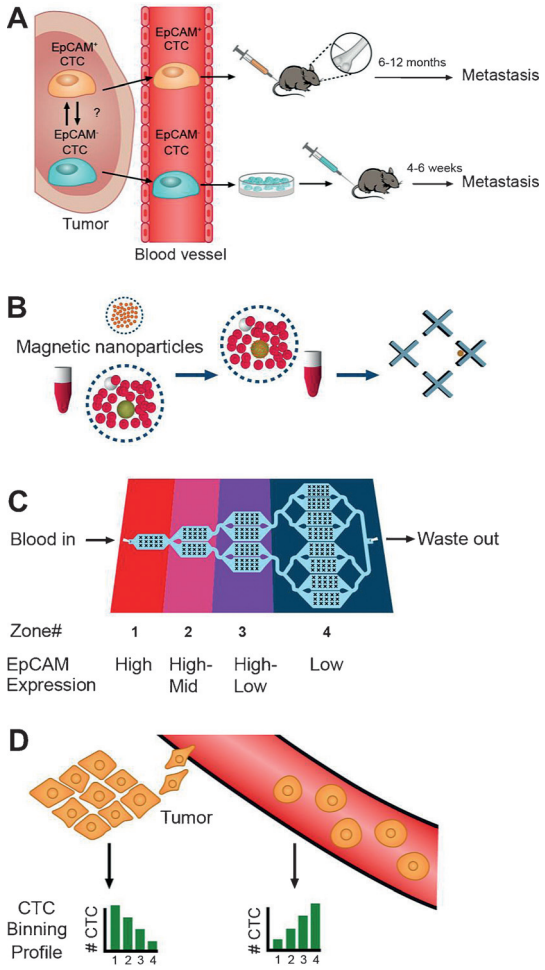

Figure 5. Phenotypically-distinct subpopulations of CTCs can be present within patient cancer samples. A) Specific subpopulations of human breast cancer CTCs have been shown to induce metastases in mice. Both EpCAM$^+$ and EpCAM$^-$ CTCs can be isolated from patients, and these cells were shown to have differing levels of metastatic potential.[86] B–D) A microfluidic approach to the isolation of subpopulations of CTCs. B) CTCs from whole blood are labeled with magnetic nanoparticles coated with anti-EpCAM antibodies. They are then captured in a microfluidic device containing microscale X-shaped obstacles that create local areas of low flow.[83] C) The microfluidic capture device sorts cells into four zones based on the levels of surface EpCAM expression. Cells with high EpCAM are trapped in Zone 1, while cells with low EpCAM are found in Zone 4.[83] D) Application of the subpopulation isolation device. CTC subpopulations have been visualized in human patients and tumor-bearing animals by using this approach.[84] Figure adapted from Refs. [83, 84, 86].

difficult to obtain in routine clinical trials or CTC culture. In addition, microfluidic devices have been used to isolate a bulk fraction of CTCs that could then be characterized on a single-cell basis.[53] None of these methods, however, are amenable to high-throughput studies of CTC heterogeneity in patient samples.

A recent advance in the separation of CTC subpopulations was realized through the creation of a microfluidic device possessing different zones that would selectively capture cells with differing levels of a surface antigen (Figure 5B, C).[83–85] CTCs labeled with magnetic nanoparticles displaying anti-EpCAM can be sorted corresponding to the levels of a surface antigen that is known to decrease during EMT. While using nanoparticles for capture is advantageous because of the very high number of binding events possible on the surface of a cell, achieving efficient capture by using these labels is a challenge because of their low magnetic susceptibility. To overcome this, microfabricated X-shaped structures were patterned within the device to create regions of low flow (Figure 5B). Zones were created within the device with decreasing linear velocities, which allowed high EpCAM CTCs to be separated from low EpCAM CTCs (Figure 5C). This approach was shown to capture low numbers of cancer cells from blood with excellent sensitivity, and was proven to be effective in separating cancer cells with varying levels of EpCAM expression.[83] The analysis of samples drawn from prostate cancer patients showed that this device could be used to effectively profile heterogeneity in CTCs by using a variety of different markers, and that the extent of EMT in patient CTCs could be elucidated. This device can also be used to monitor CTCs in animal models of cancer, thus presenting a means to better understand the dynamics of EMT (Figure 5D).[84,86]

As an alternative to events caused by single cells, CTCs could lead to metastases by breaking off from the tumor in clumps and traveling in clusters. Cell clusters are thought to initiate metastatic spread after they get stuck in capillaries.[87] Technologies such as the Cluster Chip[88] and gelatin nanocoating substrates[69] capture CTC clusters in 30% of breast cancer patients. CTC clusters are heterogeneous and include both actively proliferating cells and quiescent cells. Further work will be required to uncover the clinical significance of these approaches and the role of CTC heterogeneity in cancer progression.

3.5. Integrated Molecular Analysis of CTCs

Profiling molecular CTC biomarkers is critical to advance the use of CTC analysis in the clinic. Eventually, it would be ideal to characterize the CTC "invasome"—a collection of biomarkers that would reveal whether the cells possess invasive properties that may lead to metastases.[89] A variety of studies have pursued sequencing of patient CTCs and showed that signature mutations matching those found in a primary tumor were present in circulating cells.[64,90–92] The content of the CTC invasome, however, remains unknown. CTCs are inherently fragile cells, which complicates their analysis because it is difficult to gauge whether cells experience alterations in their molecular profile when they are transferred between devices used for capture and analysis. Deconvolving subtle differences between invasive and more benign CTCs will require that environmental conditions are strictly controlled. It is therefore attractive to integrate device functionalities to enable the characterization of CTCs without extensive manipulation of isolated cells.

Electrochemical sensors are of interest for integrated analysis devices because they are straightforward to fabricate within microfluidic devices and can be individually functionalized and addressed for multiplexed sensing. Impedance

sensors have been used for CTC identification,[31,93] and voltammetric sensing has been shown to specifically identify cancer cells.[94–97] Recently, the ability to process whole blood, capture CTCs, lyse the cells, and analyze mRNA expression by using chip-based electrochemical detectors was demonstrated (Figure 6A).[98] This entire workflow was carried out within a single integrated device and was completed within 30 min, a highly advantageous turnaround time that would help maintain the properties of CTCs and limit perturbations arising from sample manipulation.

Chip-based micro-Hall detectors (μHD) can also be used to directly profile CTCs from patient samples.[99,100] Antibody-labeled magnetic nanoparticles are incubated with whole blood containing CTCs and processed within a microfluidic device containing a μHD detector (Figure 6B). The cells are subjected to a magnetic field and take on a magnetic moment that is proportional to the number of nanoparticles captured on the cell surface. Multiple markers can be analyzed simultaneously, with different nanoparticles possessing different magnetization properties. This approach was shown to provide a powerful means to identify CTCs in clinical samples with much higher sensitivity than that achieved with CellSearch, and was used to monitor CTCs in a mouse xenograft model.

A new class of nanoparticle-based probes represents an alternative strategy for analyzing biomarkers within intact CTCs.[101] NanoFlares are created by functionalizing gold nanoparticles with thiolated oligonucleotides that are complementary to an mRNA sequence of interest. A synthetic complement with an appended fluorophore is hybridized with the immobilized sequence; the emission of this sequence is quenched as a result of proximity to the gold surface (Figure 6C). The NanoFlares readily enter cells, and if the target mRNA is present, the fluorescent oligonucleotide is liberated and generates a signal that can be used to track cells as a function of mRNA expression. This approach was applied to CTC analysis with NanoFlares specific for the mesenchymal markers Twist, Vimentin, and Fibronectin, along with the epithelial marker E-Cadherin. When the NanoFlares were used to analyze metastatic breast cancer patient samples, a specific subpopulation of cells could be visualized by using flow cytometry. This approach represents a substantial advance because it does not require any capture steps for CTC analysis and has the potential for use in identifying CTCs with a specific molecular phenotype.

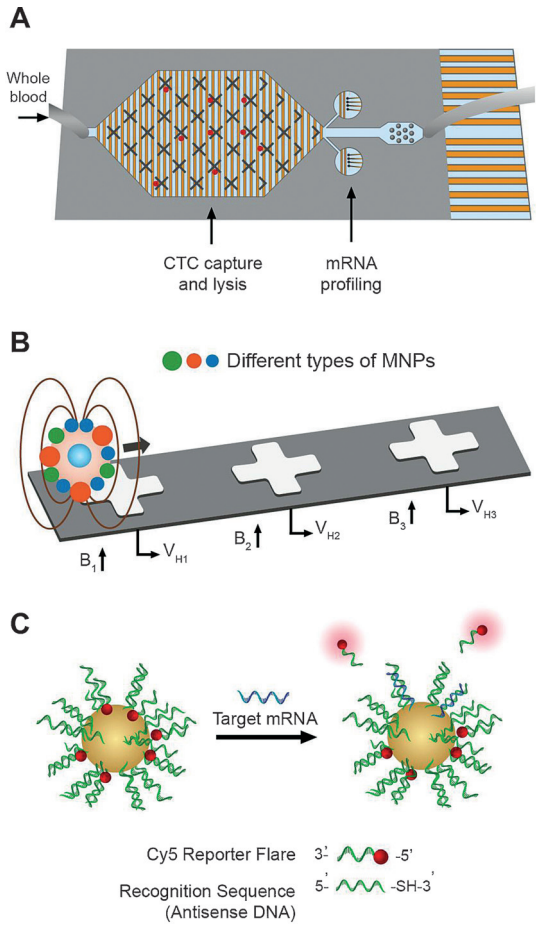

Figure 6. Systems for integrated molecular analysis of CTCs. A) CTCs bound to magnetic nanoparticles are captured in the microfluidic device, lysed on-chip, and analyzed for mRNA expression by using nanostructured microelectrodes.[98] B) The μHall chip can detect CTCs labeled with different types of magnetic nanoparticles, each targeting a different biomarker. The magnetic moments of the cells are then measured by using microfabricated Hall sensors in a spatially varying field (β1, β2, β3).[99] C) NanoFlares comprise a monolayer of antisense DNA (recognition sequence) adsorbed to the surface of a spherical gold nanoparticle. A reporter flare sequence is hybridized to the recognition sequence that contains the fluorophore (Cy5). Upon binding between the target mRNA and the complementary capture sequence, the reporter flare is displaced, thereby producing a fluorescent signal.[101] Images were adapted from Refs. [98, 99, 101].

3.6. In Situ Phenotypic Analysis of CTCs

While the development of integrated devices will facilitate the investigation of known CTC biomarkers, the complete suite of metastases-initiating factors remains uncharacterized. Therefore, methods that monitor cellular phenotypes are valuable sources of information and are progressing in parallel to biomarker assays. Motility is a critical aspect of cellular behavior that is thought to contribute to the aggressiveness of cancer cells. This behavior appears to be dependent on cell density and local environment, which makes single-cell approaches advantageous.

Recent advances in this area include the development of microfluidic devices that can be used to measure the migration of a specific mesenchymal phenotype of cells with single-cell resolution.[102] By using an array of over 3000 miniaturized chambers, migration patterns and velocities can be monitored for single cells (Figure 7A). Cultured cells treated to induce EMT were shown to have more aggressive migration phenotypes, and cells that exhibited significant levels of drug resistance showed the highest velocities. A 3D version of the microfluidic chip also permitted this behavior to be studied as a function of cell density (Figure 7B).[103] Approaches that dock micromachines with cancer cells may represent another means to analyze cellular migration.[104,105]

These types of phenotypic analyses have not yet been applied to patient CTCs, presumably owing to the challenge associated with preserving the viability of these cells. However, the continued advancement of these techniques, along with molecular profiling, may help to elucidate the factors that enhance the invasiveness of CTCs.

3.7. In Vivo CTC Analysis

While ex vivo analysis of CTCs has provided a wealth of information on the levels of these cells in different patients and the molecular profiles of blood-borne tumor cells, the ability to monitor CTCs in vivo would offer a direct means to monitor levels in real time.

This type of monitoring presents a tremendous challenge, with the high levels of background cells and the dynamics of blood-borne cells complicating the analysis. Nonetheless several approaches have shown promising progress towards in vivo CTC monitoring.

One approach to this problem relies on the injection of fluorescent ligands that specifically bind cancer cells, and the use of a flow-cytometry-like technique to monitor cells in blood vessels (Figure 8A).[106] This method was used to monitor CTCs in real time in a mouse model of metastatic cancer. Interestingly, CTCs could be detected weeks before metastatic disease was observed, indicating that this approach has a high level of sensitivity. The main limitation of this method is that significant tissue penetration could not be achieved and it is not clear whether the signals generated would be sufficient for human use.

A photoacoustic method with much deeper (ca. 3 cm) tissue penetration has also been applied to CTC analysis in vivo.[107] This approach sought to concentrate CTCs in blood vessels by using magnetic nanoparticles, and then used a two-color approach to heighten the specificity of CTC identification (Figure 8B). The ability to magnetically concentrate the CTCs in vivo was proposed to have a dual purpose, since after quantization, the local capture of the cells could be leveraged for non-invasive laser ablation.

A novel method for capturing CTCs in vivo makes use of an EpCAM-functionalized stainless steel medical Seldinger guidewire (FSMW) that is inserted into the vein of the patient for half an hour (Figure 8C,D).[108] The FSMW captured CTCs from 24 breast or lung cancer patients, and the cells were analyzed post-capture by using immunofluorescence.

Interestingly, the Google X Life Sciences group has also taken on the challenge of in vivo CTC analysis.[109] While few details are available about the effort, an interest in nanoparticles that would permit the development of an "early warning system" for cancer has been publicized.

4. Summary and Outlook

Analysis of the tumor cells that enter the circulation may eventually allow tumors to be characterized non-invasively and profiled in real time as treatment is administered and recurrence is monitored. The rarity and heterogeneous biology of these cells present significant challenges to their isolation, identification, and characterization. In the 1990s and 2000s, the first approaches emerged that permitted

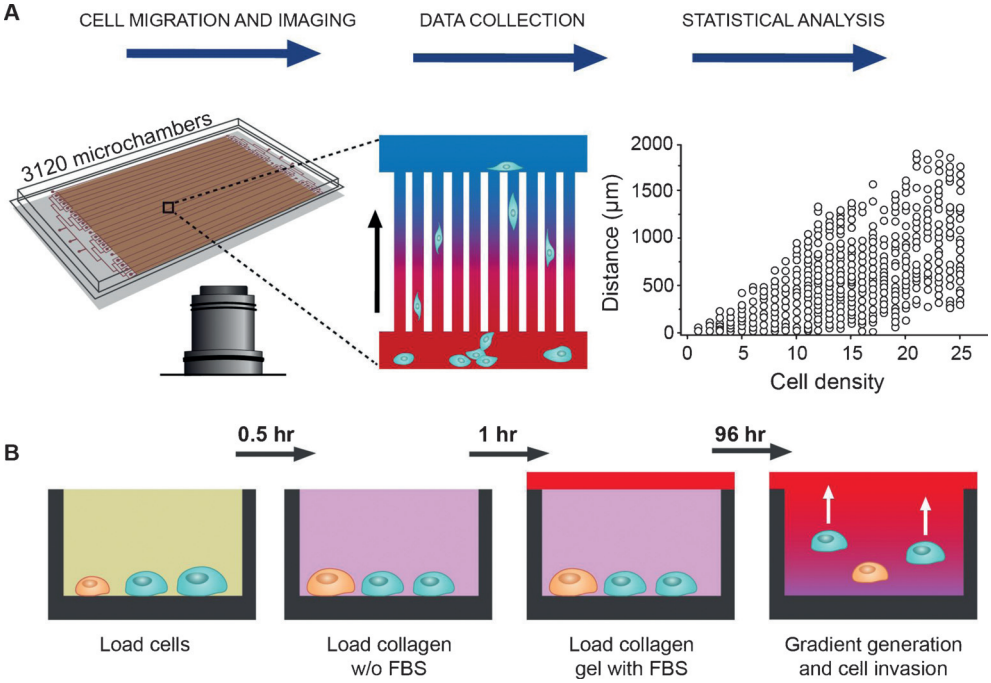

Figure 7. In situ phenotypic analysis of CTCs. A) The M-Chip is used for monitoring mesenchymal mode cell migration. Cells are plated on a basement membrane located one side of the device and migrate along microchannels towards a chemotactic agent (blue). The migration distance is shown to be dependent on the number of cells per well (density).[102] B) The MI-Chip represents a 3D cell migration assay. Cells are placed on top of a collagen gel inside miniaturized wells. Nutrients are added on top of the collagen layer. Cells move towards the nutrients (e.g., fetal bovine serum) and are tracked by using green fluorescent protein.[103] w/o = without. Images adapted from Refs. [102,103].

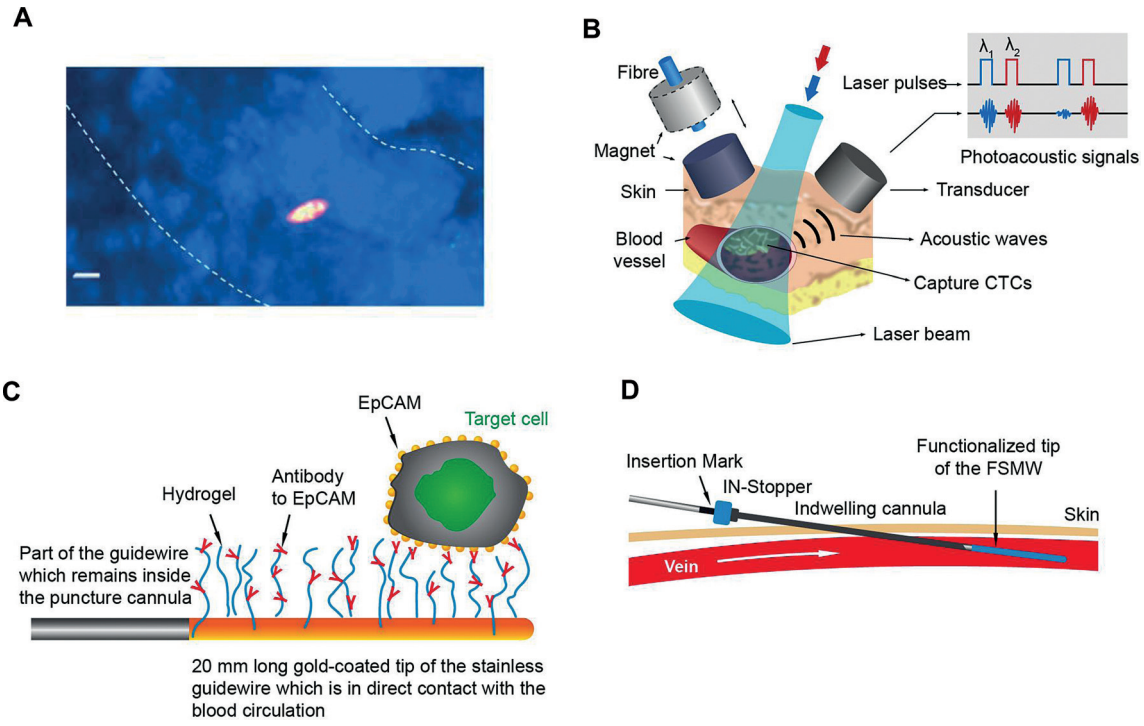

Figure 8. In vivo analysis of CTCs. A) In vivo detection of fluorescent cells in circulation by using multiphoton intravital flow cytometry. Leukemia tumor cells are labeled with folate-conjugated fluorescein isothiocyanate (FITC; green) and lipophilic tracer DiD (red). Scale bar: 10 μm.[106] B) In vivo magnetic enrichment by using two-color photoacoustic detection of CTCs. The laser beam is delivered close to the external magnet or through a hole in the magnet by using a fiber-based delivery system.[107] C) A functionalized stainless steel medical guidewire (FSMW) can be used to capture CTCs intravenously. EpCAM antibodies are conjugated to a polycarboxylate hydrogel coat on the surface of a gold-plated guidewire. D) Insertion of a FSMW into the cubital vein through a conventional cannula. The FSMW is pushed forward into the cannula until the anti-EpCAM-functionalized FSMW surface of 2 cm in length is exposed to the blood flow.[108] Images adapted from Refs. [106–108].

sensitive enumeration of CTCs in patient samples. Despite numerous studies analyzing CTC presence as a function of cancer progression, these cells have not made their way into mainstream clinical medicine as a meaningful biomarker that could be connected universally with the disease.

The new devices and materials that have emerged recently provide valuable tools that will allow more information to be extracted from these cells so that their phenotype and clinical relevance can be better understood. New systems that allow cells to be recovered and characterized at the genomic level have reinforced the idea that CTCs may represent a liquid biopsy of a tumor. Integrated microfluidic devices and new nanomaterials are permitting CTCs to be analyzed in situ to ensure that genomic and proteomic information collected reflects the true characteristics of these cells and not artifacts created during sample handling. The development of new analytical methods that allow CTCs to be monitored in vivo represents an important goal that will enable analysis under physiologically relevant conditions. Continued progress in the development of new capabilities will eventually advance our understanding of the mechanisms of cancer metastasis, which is fundamentally important for combating this disease.

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