Physical Biology in Cancer. 2. The physical biology of circulating tumor cells

Kevin G. Phillips,1,2 Peter Kuhn,3 and Owen J. T. McCarty1,4,5

1Department of Biomedical Engineering, School of Medicine, Oregon Health & Science University, Portland, Oregon; 2Department of Dermatology, School of Medicine, Oregon Health & Science University, Portland, Oregon; 3Cell Biology Department, The Scripps Research Institute, La Jolla, California; 4Division of Hematology and Medical Oncology, School of Medicine, Oregon Health & Science University, Portland, Oregon; and 5Department of Cell and Developmental Biology, School of Medicine, Oregon Health & Science University, Portland, Oregon

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Phillips KG, Kuhn P, McCarty OJ. Physical Biology in Cancer. 2. The physical biology of circulating tumor cells. Am J Physiol Cell Physiol 306: C80–C88, 2014. First published October 16, 2013; doi:10.1152/ajpcell.00294.2013.—The identification, isolation, and characterization of circulating tumor cells (CTCs) promises to enhance our understanding of the evolution of cancer in humans. CTCs provide a window into the hematogenous, or “fluid phase,” of cancer, underlying the metastatic transition in which a locally contained tumor spreads to other locations in the body through the bloodstream. With the development of sensitive and specific CTC identification and isolation methodologies, the role of CTCs in clinical diagnostics, disease surveillance, and the physical basis of metastasis continues to be established. This review focuses on the quantification of the basic biophysical properties of CTCs and the use of these metrics to understand the hematogenous dissemination of these enigmatic cells.

Cancer metastasis to distant sites from the originating organ is the leading cause of cancer-related deaths and is thought to be facilitated by the hematogenous transport of circulating tumor cells (CTCs). Profiling of CTCs provides an opportunity to noninvasively diagnose, monitor, and elucidate the evolution of cancer as it transforms from a locally contained mass into a disseminated disease spreading to multiple locations in the body (24, 43). While this endeavor is now over 100 years old (47), only recently have investigators made translational gains in the development of new technologies to identify, characterize, and isolate CTCs for enumeration, functional assessment, and exploration of biomarkers indicative of disease type and treatment response. In addition to potential disease surveillance applications, modern CTC isolation technologies are providing a crucial means to enable the quantification of the basic physical features of cancer cells in the bloodstream.

Known to be a rare event with yet-to-be-determined biology, the isolation and characterization of CTCs from whole blood have been impeded owing to their extreme rarity. To overcome this challenge, modern isolation strategies exploit physiochemical differences among CTCs and the background population of platelets, red blood cells (RBCs), and white blood cells (WBCs) in the circulation. These approaches include immunolabeling of specific epithelium-specific proteins with fluorescent antibodies (32, 33) or magnetic beads (3), functionalization of microfluidic surfaces to specifically capture epithelial cells (51), and utilization of the distinguishing physical properties of CTCs, e.g., density-based gradient centrifugation or size-based filtration (19), to facilitate their detection.

Recent findings in the investigation of CTCs have been distilled into several reviews on the physical interactions and mechanical forces involved in metastasis (56, 60), the clinical utility of CTCs (42), the role of CTCs in coagulation and thrombosis (53), and the engineering principles underlying current CTC capture methodologies (62). The primary data from the field and these reviews highlight the vast physical diversity of potential disease-derived cells and the resulting complexity in analysis and clinical interpretation. Many debates continue around the following questions 1) What is the CTC? 2) What are the non-CTC rare cells that are present in patients? The current review highlights contemporary efforts to directly quantify the basic physical features of candidate CTCs.

We highlight investigations of the physical parameters of the range of potential disease-derived CTCs, the isolation platforms enabling these measurements, the phenotypic bias inherent in these platforms, and the role these metrics play at the interface of fluid mechanics, coagulation, and adhesion mechanisms underlying the metastatic cascade. Reliable analytic methods enabling single-cell analyses will contribute to the understanding of these highly heterogeneous cell populations.1

Capture and Isolation Methodologies

All CTC platforms require a sample preparation process (e.g., centrifugation, filtration, staining, and culture) followed by sample analysis (e.g., imaging, RT-PCR, and transplantation) followed by data analysis. Fine-tuning the overall rela-

1 This review is part of a five-article theme series on Physical Biology in Cancer in this issue.
tionship of these steps from beginning to end is central to the successful evaluation of disease-derived CTCs.

Different approaches will produce alternate results in settings addressing different questions. For example, if one is interested in looking for patients with more than one epithelial cell adhesion molecule-positive cell per milliliter of blood, then CellSearch is appropriate (3). If one is interested in investigating cells larger than the leukocyte population, then filters are sufficient (18). Alternatively, if one wants to characterize the largest diversity of candidate disease-derived CTC populations, then an enrichment-free approach is warranted (33).

No single assay is capable of tuning its sensitivity from a “fine” to a “coarse” approach to CTC capture. Hence, the successful evaluation of concentration, morphology, subcellular structure, protein expression profiles, and function requires the balancing of multiple assay performance constraints, including processing speed, maintenance of cell viability, capture sensitivity/specificity, and measurement stability, to meet the quantitative goal desired. Here we focus on assays enabling the use of fluorescence and light microscopy to evaluate the basic physical features of CTCs.

**Density-based gradient centrifugation as minimal enrichment.** Centrifugation remains an inexpensive and simple means to fractionate blood into its constituent cellular components. Separation is achieved by the application of centrifugal forces to the sample, with cellular size and density determining the level of fractionation of the sample. In general, larger and denser particles pellet at lower centrifugal force (~100 g), and smaller and less dense particles fractionate at very high centrifugal force (~1,000 g). Centrifugation applied to whole blood results in the fractionation of the cellular constituents into three distinct layers: plasma on the top layer, accounting for ~55% of whole blood; nucleated cells consisting of WBCs (5 × 10⁹/ml), platelets (2 × 10¹⁰/ml), and CTCs (0–800/ml) on the middle layer; and RBCs (8 × 10¹²/ml) on the bottom layer.

As early as 1917, centrifugation, in combination with hemolysis, was utilized to separate CTCs from background blood cell populations (31). Cells from the nucleated layer were fixed, dehydrated, and then embedded in paraffin. Multiple sections were cut at varying intervals through the block, stained, and mounted under a coverslip for visualization under a microscope. In 1934, Pool and Dunlop (47) used this technique to detect CTCs in 42% of patient samples obtained from 40 patients with a variety of advanced carcinomas (Fig. 1A).

Today, “enrichment-free” centrifugation is still utilized by investigators, as this approach does not require specific size- or protein-based assumptions. In the high-definition CTC (HD-CTC) assay, Marrinucci et al. (33) retained all nucleated cells from fractionated whole blood and dispersed these cells as a monolayer on glass slides. Cells were subsequently stained with immunofluorescent monoclonal antibodies targeting cytoketin (CK), an intermediate filament found exclusively in epithelial cells, a pan-leukocyte-specific antibody targeting CD45, and a nuclear stain, 4',6-diaminido-2-phenylindole (DAPI). Automated fluorescence imaging was then performed to identify CTCs, classified as being CK-positive and CD45-negative, with an intact nonapoptotic-appearing nucleus detected by 4',6-diaminido-2-phenylindole staining (Fig. 1, D–F). This method detected CTCs in 70% of 30 metastatic breast cancer patients, 80% of 20 metastatic prostate cancer patients, and 50% of 18 metastatic pancreatic cancer patients. With no size or chemical constraints on isolation, the advantage of this approach is that it is not likely that CTCs will be inadvertently lost during processing.

**Density-based gradient centrifugation in conjunction with filtration.** Secondary approaches applying size-based filtration are able to separate CTCs from contaminating WBC populations. In 1959, Seal (49) improved centrifugation approaches to CTC purification through the introduction of silicone oils to whole blood that, with the application of centrifugal forces, separated the WBC population into mononuclear (monocytes and lymphocytes) and polymorphonuclear (neutrophils, eosinophils, and basophils) fractions (49). Mononuclear cells, including CTCs, as well as platelets, were fractionated together above the silicone oils, while RBCs and polymorphonuclear leukocytes were sequestered below the oil layer. Interestingly, this method captured CTC aggregates in the mononuclear fraction. Cells from the mononuclear fraction were filtered through a porous membrane containing 5-μm-diameter pores. Cells were stained and imaged directly on the filter, enabling the detection of CTCs in 45% of 86 patient samples (Fig. 1B).

Modern efforts to combine filtration with centrifugation are employed in the OncoQuick assay. This purification modality replaces the silicone oils used by Seal (49) with a porous filter that allows RBCs and some WBCs to pass through, and CTCs are sequestered above the barrier. This technique achieved 632-fold enrichment against leukocytes compared with 3.8-fold enrichment with traditional centrifugation alone (48). While isolation of candidate CTCs larger than the WBC population is enhanced, cells of comparable size to the leukocyte population are potentially lost.

**Microfiltration.** Microfiltration technology uses high-throughput filtration of whole blood through polycarbonate track-etch-type membranes to separate normal blood cell constituents from CTCs. In the isolation by size of epithelial tumor cells (ISET) assay, a 600-μm² membrane with calibrated 8-μm-diameter cylindrical pores is used to filter blood cell constituents (57). Blood is first diluted 1:10 in filtration buffer, a mixture containing EDTA, saponin, bovine serum albumin, and paraformaldehyde, and then drawn through the filter under gentle vacuum. The membrane is disassembled from the filtration module, allowed to air-dry, and then stained with hematoxylin and eosin for microscopic investigation (Fig. 1C). ISET detected CTCs in 95% of 60 patients with metastatic breast, prostate, and lung cancer (16). ISET platforms designed around the maintenance of cell viability have also been developed. The membranes possess support structures to reduce mechanical stresses on CTCs. The ScreenCell platform is capable of 74–91% recovery and 85% viability (15). As with the OncoQuick approach, the cells of comparable size to the leukocyte population are potentially lost.

**Microfluidics.** With their ability to control fluid behaviors over a range of scales, microfluidic devices enable highly efficient processing of complex cellular fluids with minimal damage to cell populations arising from fluid shear forces. Surface functionalization coupled with the complex flow geometries, disrupting laminar streamlines, such as by the generation of microvortices, enables cellular interactions with antibody-coated surfaces for CTC capture.

The micropost CTC-Chip relies on laminar flow disruption by anti-epithelial cell adhesion molecule antibody-coated microposts (39), while the herringbone CTC-Chip (HbCTC-Chip)
generates microvortices via herringbone-shaped grooves to direct cells toward antibody-coated surfaces (51). HbCTC-Chip detected CTCs in 14 of 15 (93%) patients with metastatic disease (median \(63\) CTCs/ml, mean \(386\) CTCs/ml).

The recent inertial focusing CTC-Chip (CTC-iChip) combines deterministic lateral displacement, i.e., sorting with microposts based on cell size, to separate nucleated cells in whole blood from RBCs and platelets, with inertial focusing of nucleated cells to flow in a single-file line (41). This integrated platform has the ability to isolate CTCs from whole blood using tumor antigen-dependent and -independent modes and is compatible with high-definition imaging (Fig. 1, G–I) to allow for standard clinical cytopathology. The CTC-iChip detected CTCs in 37 of 41 (90%) prostate cancer patients with recurrent (castration-resistant) disease (mean \(50.3\) CTCs/ml, range \(0.5–610\) CTCs/ml, median \(3.2\) CTCs/ml).

**Quantification of CTC Biophysical Properties**

The integration of measurement technologies into CTC isolation and capture assays remains challenging, as many assays sequester cells in device compartments that are typically amenable only to light microscopy. Moreover, the identification of CTCs in the device often involves cellular fixation to enable transport and membrane permeabilization (required of CK staining), processes that make assessment of the “true”
physiological state of CTCs impossible. These tools used to identify cells present a “rare cell uncertainty principle,” synonymous with the Heisenberg uncertainty principle of quantum mechanics, in that the more we know about where a specific cell is (position information), the less we can study its true biology (dynamic information) in its true physiological state.

The newly discovered microfluidic strategies that can identify CTCs independently of tumor membrane epitopes, such as the CTC-iChip (41), can be potentially used to load viable CTCs into instruments capable of single-cell biophysical measurements. These technologies include atomic force microscopy to probe cell stiffness; traction force microscopy to monitor traction stresses exerted by cells through focal adhesions; ballistic intracellular nanorheology to monitor local viscoelastic properties of the cytoplasm; a suspended microchannel resonator to measure cellular mass, volume, and density in solution, as well as deformability and surface friction; and a microfluidic optical stretcher to measure cellular elasticity (2, 9, 11, 18).

As technologies aimed at maintaining CTC viability continue to come online and the compatibility of these assays with biophysical quantification continue to be developed, alternative strategies employing novel uses of light microscopy are being pursued to characterize CTCs in the current isolation platforms. These approaches include quantitative phase microscopy (QPM) to quantify cellular area, mass, and density (44, 46); Hilbert transform differential interference contrast (DIC) microscopy (HTDIC) to quantify cellular volume (6, 45); fluorescence microscopy to assess area, nuclear-to-cytoplasmic ratio, and diameter (25, 51); and standard bright-field microscopy to investigate cellular area and diameter (41). As cellular preparations vary in the use of fixatives, permeabilization agents, and staining protocols, it is critical to compare CTC metrics with WBC metrics under the same experimental conditions used to facilitate CTC isolation.

**Area and diameter.** Cellular diameter and area are readily quantified, albeit biased by sample preparation, through calibrated microscope systems employing digital imaging and modest image segmentation algorithms, such as the ImageJ software package (1) (Table 1).

In a study by Lazar et al. (25), prostate CTCs were isolated and characterized using the HD-CTC assay. Cytometric features of prostate CTCs were compared with the prostate cancer cell line LNCaP (Fig. 2D). Prostate CTC mean areas ranged from ~50 to ~130 μm², while LNCaP cell areas were ~150 ± 50 μm². In a separate HD-CTC study of CTCs identified in an ovarian cancer patient (Fig. 2, A and B), CTC area was 138 ± 4μm², with a background leukocyte population of 51.8 ± 8.3 μm² (46). In a study of CTCs in a breast cancer patient, HD-CTC area was 138 ± 38.8 μm², while the leukocyte population was 48.0 ± 3.8 μm² (45).

In a study employing the HbCTC-Chip (51), prostate cancer CTCs displayed variable diameters ranging from 8 to 16 μm; the lower (8-μm-diameter) limit of CTCs observed in this

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Value</th>
<th>Isolation Platform</th>
<th>Measurement Modality</th>
<th>Ref. No.</th>
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<td>HD-CTC</td>
<td>Fluorescence microscopy</td>
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<td>HD-CTC</td>
<td>DIC microscopy</td>
<td>45</td>
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<td>DIC microscopy</td>
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<tr>
<td>Prostate CTC</td>
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<td>HD-CTC</td>
<td>HTDIC microscopy/NIQPM</td>
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Values are means ± SD, unless otherwise noted. CTC, circulating tumor cell; HD-CTC, high-definition CTC; HbCTC, herringbone CTC Chip; CTC-iChip, inertial focusing CTC-Chip; DIC, differential interference contrast; HTDIC, Hilbert transform DIC; NIQPM, noninterferometric quantitative phase microscopy. *Patient-matched to CTC.
study is comparable to the median size of leukocytes and corresponds to a circular area of 50 μm².

Using the CTC-iChip, Ozkumar et al. (41) observed large variations in CTC size among different cancer types. Although some CTCs were larger than leukocytes, there was considerable overlap between the two cell populations in a melanoma (Fig. 2H) and a breast cancer (Fig. 2I) patient. Although most melanoma CTCs were large (>12 μm diameter; Fig. 2G), in one patient with metastatic melanoma, numerous <10-μm-diameter CTCs were detected using Papanicolaou staining and immunocytochemistry for Melan-A.

**Mass, volume, and density.** The optical quantification of cellular dry mass content/density and volume of CTCs has been carried out on CTCs isolated in the HD-CTC assay using QPM to quantify mass/density and HTDIC to quantify volume (41, 45, 46) (Table 1). These optical approaches are employed on a conventional microscope to perform optical sectioning of the cells of interest at low-numerical aperture (NA) illumination under bright-field contrast (QPM) and high-NA illumination under DIC contrast (HTDIC).

QPM is a method used to reconstruct the phase of optical waves traveling through the sample (Fig. 2C). Phase is linearly related to the axially integrated mass density of the cellular specimens (44). Summation of this axially integrated mass density over the area of the specimen gives the total dry mass content of the specimen. Volumetric information can be obtained from through-focus DIC imagery under high-NA illumination conditions (Fig. 2B) that enable optical sectioning of the specimen along the optical axis. Hilbert transform processing on the DIC image stacks greatly enhances edge-detection algorithms for localization of the specimen borders in three dimensions by separating the gray values of the specimen intensity from those of the background. Fourier filtering methods to enhance contrast and a Sobel-based edge-detection method enable automated volumetric analysis of the sample (6). QPM and HTDIC have been validated on engineered polystyrene spheres ranging in size from the diffraction limit up to 20 μm diameter (46).

In a study of breast cancer-associated CTCs (45), the average measured volume of CTCs was 851 ± 45.8 μm³ (n = 42 cells), and the leukocyte population was 234.1 ± 4.1 μm² (n = 100 cells). In an HD-CTC study of 31 CTCs from an ovarian cancer patient, QPM and HTDIC (46) revealed an average CTC volume of 518.3 ± 24.5 μm³, mean dry mass content of 33.6 ± 3.2 pg, and mean density of 0.065 ± 0.006 pg/fl. In the leukocyte population (n = 50 cells) from the same patient, mean volume was 230.9 ± 78.5 μm³, mean mass was 18.7 ± 0.6 pg, and mean density was 0.085 ± 0.004 pg/fl.

**CTC Biochemical Characterization**

During their transit in the circulation, CTCs are exposed to blood coagulation factors, plasma proteins, and cells, such as platelets and leukocytes, all of which may affect their survival and metastasis (10, 23, 36, 37). Moreover, metastatic cancer
Patients are known to be at a greatly increased risk for thrombotic complications, such as venous thromboembolism (50), suggesting that the interplay between CTCs and the blood microenvironment may have pathological consequences (53). However, it is unclear whether the link between thrombosis and cancer is due to the prothrombotic action of CTCs. Tumors and cancer cell lines have been shown to express the transmembrane glycoprotein tissue factor (TF) (7, 28, 52). TF is the primary initiator of blood coagulation and is expressed by hematopoietic cells (monocytes) and a variety of nonhematopoietic cells (endothelial cells, epithelial cells, smooth muscle cells, and fibroblasts) (29). TF complexes with the coagulation factor VII to activate coagulation factors IX and X, leading to thrombin generation (8). This process is localized to the cell surface by phosphatidylserine exposure (30). In vitro, the biophysical parameters of TF expression and extent of phosphatidylserine exposure have been shown to regulate the procoagulant activity of cancer cell lines (7). Moreover, recent studies have shown that the coagulation kinetics for circulating TF are sensitive to the spatial separation of TF carriers (the average distance between TF carriers in suspension) (55) and the variation of coagulation factor IX and X levels within the physiological range (54). Along these lines, elevated levels of circulating TF-expressing microparticles have been shown to be associated with thrombosis in cancer (40, 64). Moreover, recent in vitro studies have highlighted the role of coagulation in recruitment of circulating colon adenocarcinoma cells to thrombi in a factor Xa- and shear-dependent manner (5).

The biochemical signature of CTCs has yet to be characterized. In particular, it is unknown whether CTCs express TF, in an “active” or a “decrypted” form. Efforts are underway to develop fluorescently labeled coagulation factors to characterize the procoagulant signature of CTCs, which may provide a method to gain insight into the role of CTCs in the development of thrombosis in patients with cancer.

**Numerical Models of CTCs in Blood Flow**

The numerical encoding of the dynamic interactions of CTCs with blood cell constituents, endothelial cells, blood flow geometries, and coagulation factors in a comprehensive theory of CTC dissemination through the vasculature remains an ongoing challenge in the quantitative characterization of metastasis. Fortunately, many of the tools utilized to model blood cell interactions, such as platelets and leukocytes (20, 38), in the circulation can be adapted to the study of CTCs. For instance, the interactions of blood cells with the vasculature are well developed: the sizes of all the cellular constituents of blood are known (17), and the receptor-ligand on-off kinetics enabling adhesive interactions with the endothelium are well characterized (10, 26, 58), as are the fluid pressures driving blood flow in the arteriole and the venous circulation (14). With the ability to quantify CTC biophysical properties, researchers can begin to incorporate new information into models of cellular fluid mechanics. Two integral facets of a model of CTC dynamics in the circulation are the role of coagulation and the role of adhesion molecules.

Lee et al. (27) employed a coupled model of fluid dynamics and concentration field equations to investigate thrombin concentration gradients generated by procoagulant CTCs in flow. The principal findings consist of build-up of concentration fields near vessel walls, near regions surrounding CTCs, and in complex time-dependent regions of the flow where fields associated with different CTCs overlap. Figure 3A demonstrates a temporal evolution of thrombin generation propagating away from CTCs under uniform flow conditions. This study models CTCs as point particles; hence, the physicality of CTCs is not incorporated into the investigation. With the quantitative characterization of CTC volume and density, the interaction of CTCs in blood flow can be incorporated in the model to examine the role of CTC geometry in driving the procoagulant properties of CTCs near the vessel wall and in the microcirculation.

To assess leukocyte-endothelial interactions, Chang et al. (12) investigated the role of bond dissociation rates and the spatial dependence of potential functions determining bond forces to model selectin interactions between WBCs and endothelial cells. Interestingly, distinct physical states, including firm adhesion, rolling adhesion, and no adhesion, were observed in the parametric phase space of the model (21, 22). These regimens were validated experimentally (Fig. 3B). As CTCs possess complex geometries that will affect their translation and angular velocities under flow, a complex slipping behavior that increases the residence time of receptor-ligand...
pairs to facilitate adhesion processes is likely to occur (13). It is thus expected that models of CTC-endothelial cell interactions will predict different regimens of firm and rolling adhesion that will be influenced by CTC volume and, hence, surface area, as well as density-mediated deformation arising from flow interactions. Models incorporating stochastic realizations of CTC size, as well as both adhesion and coagulation mechanisms, will help unravel the complex nature of CTC transit, arrest, and metastatic capacity.

Conclusions

The primary goal of CTC identification assay development has been the correlation of clinical metrics of a patient’s disease to new forms of information present in CTCs. This includes the CTC concentration in the circulation (3), morphology (34, 35), size (33), mass (46), volume (45, 46), density (46), density variations (45), epithelial-to-mesenchymal transition markers (61), DNA damage (59), RNA signatures (41, 51), and functional assessment of tumorigenic potential (4, 63). Research efforts are underway to define the rate and route of CTC entry into the blood from the primary tumor and the lifetime of CTC transport within the vasculature and to determine the influence of the blood microenvironment on the genetic, epigenetic, and proteomic profiles of CTCs.

Understanding the fluid phase of solid tumors is as challenging as understanding the solid phase, which to this day does not have quantitative descriptors based on first-principle physical models. With the increasing awareness that enhancing our understanding of the physical origins of cancer will rely on a multifaceted set of measurements to probe the parametric “phase space” of normal and pathological tissue function (2), the varied CTC isolation platforms provide orthogonal measurements of CTC function and cellular structure that, taken together, provide a new knowledge base from which to model and understand CTCs and their dynamic role in metastasis. New biophysical insights into CTC physical properties will empower first-principles approaches to model metastasis and potentially identify parameters that can be manipulated through therapeutic interventions.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

K.G.P. prepared the figures; K.G.P., P.K., and O.J.M. drafted the manuscript; K.G.P., P.K., and O.J.M. approved the final version of the manuscript; P.K. and O.J.M. edited and revised the manuscript.

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