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Dynamic physical properties of dissociated tumor cells revealed by dielectrophoretic field-flow fractionation

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Abstract

Metastatic disease results from the shedding of cancer cells from a solid primary tumor, their transport through the cardiovascular system as circulating tumor cells (CTCs) and their engraftment and growth at distant sites. Little is known about the properties and fate of tumor cells as they leave their growth site and travel as single cells. We applied analytical dielectrophoretic field-flow fractionation (dFFF) to study the membrane capacitance, density and hydrodynamic properties together with the size and morphology of cultured tumor cells after they were harvested and placed into single cell suspensions. After detachment, the tumor cells exhibited biophysical properties that changed with time through a process of cytoplasmic shedding whereby membrane and cytoplasm were lost. This process appeared to be distinct from the cell death mechanisms of apoptosis, anoikis and necrosis and it may explain why multiple phenotypes are seen among CTCs isolated from patients and among the tumor cells obtained from ascitic fluid of patients. The implications of dynamic biophysical properties and cytoplasmic loss for CTC migration into small blood vessels in the circulatory system, survival and gene expression are discussed. Because the total capacitance of tumor cells remained higher than blood cells even after they had shed cytoplasm, dFFF offers a compelling, antibody-independent technology for isolating viable CTCs from blood even when they are no larger than peripheral blood mononuclear cells.

Introduction

Metastatic disease, the major cause of poor outcome in many cancers, results from the shedding of cells from a primary tumor, their transport through the cardiovascular system as circulating tumor cells (CTCs), and their engraftment and growth at distal sites in the body as new, often more aggressive tumors^{1,2}. Considerable energy is being focused on developing better methods to isolate and characterize rare CTCs from the peripheral blood^{2,3} because they have been shown to be of prognostic value² and, more importantly, may facilitate diagnosis and treatment planning by revealing molecular information about the primary tumor from a simple blood draw^{3,4}.

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Conflict of Interest Disclosure

PRCG holds interests in several patents on DEP-FFF that are under license to ApoCell, Inc, and ApoCell is providing support to his laboratory for technology development and to him in a consulting capacity.

Little is known about how cancer cells change after they detach from their growth site and undertake the journey as CTCs in the challenging environment of the circulatory system. Cell structure-function relationships depend on complex synergies between intrinsic morphological and gene expression characteristics and extrinsic mechanical and molecular interactions with the milieu^{5,6}. Consequently, the form and function of cancer cells may be expected to alter when they detach from their growth site and enter the circulation. If they are sufficiently adaptable, they may survive and function; on the other hand, if the stresses are too great, they may suffer functional impairment or death through apoptosis⁷ or anoikis⁸ in a process termed metastatic inefficiency.

In this article we apply *analytical dielectrophoretic field-flow fractionation*⁹ (dFFF) to profile cell density, capacitance, membrane area, hydrodynamic properties, and deformability of cultured cancer cells after detachment from their growth environment and maintenance as single cells in suspension as a model for CTCs newly shed from a tumor. We compare their physical properties and morphological characteristics to those of peripheral blood mononuclear cells (PBMNs) and erythrocytes under similar conditions.

The properties of freshly freed cancer cells are shown to differ significantly from those of PBMNs and to exhibit time-dependent changes in biophysical properties and morphology as a result of cytoplasmic loss to produce vesicles. Human ovarian cancer cells shed into the peritoneal cavity *in vivo* show a similar morphological profile to cultured cells that have stood in suspension for several hours following harvest, and also exhibit ongoing cytoplasmic loss, suggesting that this process is not limited to cultured cells. We suggest that the spectrum of CTC morphologies reported in the literature within individual clinical specimens may reflect tumor cells that have undergone different degrees of cytoplasmic loss. The implications of the tumor cell biophysical properties and cytoplasmic shedding to survival, migration through blood vessels, engraftment and gene expression, and to microvesicles in the circulation are discussed.

dFFF^{10–13} offers a cell surface marker-free approach to rare cell isolation^{14–16} that discriminates between cells on the basis of their total membrane capacitance, which is proportional to their cell membrane surface area. We confirm that cell capacitance provides a strong basis for detecting and isolating cancer cells from blood by dielectrophoresis (DEP).

Background to Biophysical parameter extraction by dFFF

In dFFF, cells are positioned in a hydrodynamic flow profile using sedimentation, dielectrophoretic and hydrodynamic lift forces^{9,17,18}. When cells are injected onto the inlet end of a dFFF chamber, become positioned at equilibrium heights above the chamber floor (so-called hyperlayer mode FFF¹⁹), and are carried downstream, the force balance may be written

$$F_{sed} + F_{DEP} + F_{HDL} = 0 \quad (1)$$

Then, knowing the hydrodynamic velocity profile, the heights at which this force balance has occurred may be inferred from the cell velocities determined from the cell transit times through the dFFF chamber. On the other hand, if cells come into contact with the floor of the chamber (so-called steric mode FFF¹⁹), then they experience drag forces at that will greatly slow their elution or may even stop them completely.

The goal in analytical dFFF is to measure cell elution characteristics under several experimental conditions selected to allow the individual contributions F_{sed} , F_{DEP} and F_{HDL} to be isolated from one another so that the cell biophysical properties underlying them can

be deduced⁹. The cellular properties that may be determined in this way are the density, the “crossover frequency” f_0 that reflects cell membrane capacitance (see later), and cell mechanical deformability factor in a shear flow. The parameters used to analyze the cell behavior in dFFF are defined in Table 1.

The sedimentation force F_{sed} is given by

$$F_{sed} = \frac{4}{3}\pi \cdot R^3(\rho_p - \rho_s)g \quad (2)$$

The DEP force may be written (adapted from ref 17) as

$$F_{DEP} = 352\pi\epsilon_s\epsilon_0 d^{-3}(P(f) \cdot V)^2 \exp\left(-\frac{4\pi h}{d}\right) \cdot R^3 \cdot f_{CM}(f) \quad (3)$$

when it is produced by applying an AC voltage to a periodic array of plane, parallel microelectrodes patterned on the chamber floor. The factor $P(f)$ takes into account voltage losses that lower the electric field in the fluid above the electrodes due to frequency-dependent electrode polarization¹⁷ as well as stray impedances in the leads and bus lines. The real part of the Clausius-Mossotti factor^{10,11} $f_{CM}(f)$ describing the net dielectric properties of the cells in the eluate may be approximated for mammalian cells when $1 \text{ kHz} < f < 1 \text{ MHz}$ and when the eluate has a much lower conductivity than the cytoplasm as²⁰

$$f_{CM}(f) = \left[\frac{f^2 - f_0^2}{f^2 + 2f_0^2} \right] \cdot \quad (4)$$

To maintain a high differential conductance between the interior and exterior of the cell, the cell membrane barrier must be intact so that Eqn 4 applies specifically to viable cells. f_0 is a characteristic crossover frequency at which the cell exhibits a null DEP response in an eluate of given conductivity^{13,21}. A related characteristic that is independent of the eluate is

$\Theta_0 = \frac{f_0}{\sigma_s}$, the cell crossover frequency per unit conductivity of the suspending medium. For a cell having a small membrane conductivity, the membrane capacitance per unit area C_{mem} and cell total capacitance C_{tot} , respectively, may be written simply as

$$C_{mem} = \frac{1}{2^{\frac{1}{2}}\pi \cdot R \cdot \Theta_0} \text{ and } C_{tot} = \frac{2^{\frac{3}{2}}R}{\Theta_0} \cdot \quad (5)$$

. The hydrodynamic lift force acting on cells at low Reynolds numbers may be approximated using the expression derived for deformable lipid vesicles^{22,23} as

$$F_{HDL} = -\eta\dot{v}_0 \frac{R^3}{h} \cdot \Phi \quad (6)$$

where $\dot{v}_0 = \frac{6B}{H^2W}$ is the flow shear rate at the chamber floor and B is the eluate flow rate through the dFFF chamber. Φ is a dimensionless geometry function ($0 < \Phi < 1$) that describes the deviation from sphericity of the cell^{22,23} under flow conditions. This parameter reflects the combined influence of initial shape and deformability under shear.

Because all the force components in Eqn 1 depend on R^3 , the force balance equation may be written as

$$a(\rho_p - \rho_s) + b \cdot P(f) \cdot V^2 \cdot \exp\left(-\frac{4\pi h}{d}\right) \cdot \left[\frac{f^2 - f_0^2}{f^2 + 2f_0^2}\right] - c \cdot \frac{B \cdot \Phi}{h} = 0, \quad (7)$$

where a , b , c and d are known constants for the apparatus, ρ_s , V , f and B are the known experimental conditions, and the transit height h is deduced from the cell velocity v_p derived from the observed cell elution time. Empirical equations²⁴ allowing h to be deduced from v_p are:

$$\frac{v_p}{R\dot{v}_0} = \frac{0.7431(1+h/R)}{0.6376 - 0.2000\log(h/R)} \text{ for } h \ll R;$$

$$\frac{v_p}{R\dot{v}_0} = (1+h/R) \cdot \left[1 - \frac{5}{16} \left(\frac{1}{(1+h/R)}\right)^3\right] \text{ for } h \gg R,$$

and, conveniently, the blended function derived from these,

$$v_p(h) = \dot{v}_0 R (1+h/R) \cdot \min\left(\frac{0.7431}{0.6376 - 0.2000\log(h/R)}, 1 - \frac{5}{16} \left(\frac{1}{(1+h/R)}\right)^3\right), \quad (8)$$

is continuous and may be used to derive h from the elution time computationally⁹.

To separate the cell parameters ρ_p , f_0 and Φ and to derive C_{tot} and membrane area, dFFF elution profiles for the cells were measured under three different experimental regimes:

Low frequency regime

when $f \ll f_0$ the DEP force is maximally repulsive and essentially independent of cell properties. Furthermore, at the DEP voltages used under our experimental conditions, cells are levitated high enough above the chamber floor ($>25 \mu\text{m}$) that F_{HDL} becomes negligible and the force balance equation may be approximated as $F_{sed} + F_{DEP} = 0$ at low flow rates ($B < 1 \text{ mL} \cdot \text{min}^{-1}$). In this case, the only cell unknown affecting transit time through the chamber is the cell density. Therefore, in this regime, the elution profile for a cell population may be mapped directly to the corresponding cell density profile. For experiments conducted here, a DEP operating frequency of 15 kHz was used for measuring cell densities.

No DEP regime

when $V = 0$ the DEP force is zero and the chamber operates according to sedimentation FFF principles^{19,25}. At high enough flow rates, the hydrodynamic lift force ensures that cells transit the chamber above, and in the absence of steric interactions with, the floor of the chamber and the force balance equation may be approximated as $F_{sed} + F_{HDL} = 0$. The deformability of the cell plays an important role in determining F_{HDL} , and the dependency of the cell elution time on the eluate flow rate can be used, in combination with the cell density information obtained in (1), to characterize the cell deformation characteristics. In this study, cell elution times were measured at 6 and 10 $\text{mL} \cdot \text{min}^{-1}$ and the mean cell elution time was plotted against the square of the flow rate.

Swept frequency regime

when $f \gg f_0$, the DEP force is strongly attractive and cells are pulled to the chamber floor, imposing steric forces that are strong enough to effectively halt their transit through the chamber. If the frequency is decreased steadily with time, the DEP force will gradually decrease until, as $f \rightarrow f_0$, the force will be small enough for them to be released and transported along the floor of the chamber by the eluate. As the sweep continues, the cells become more levitated by a repulsive DEP force and travel faster in the hydrodynamic flow profile until, as $f \ll f_0$ they become maximally levitated and are carried rapidly the remaining length of the chamber. In this swept-frequency regime, the total transit time through the chamber reflects how long a cell has to wait before the frequency reaches $f \approx f_0$ and the height to which it is levitated when $f \ll f_0$ (determined in the low frequency regime). In this study, the DEP frequency was swept from 300 kHz to 15 kHz.

Experimental

Cells

MDA-MB-435 and MDA-MB-231 cells were obtained from ATCC and cultured at 37°C under a 95% air/5% CO₂ atmosphere in T25 flasks containing 10 mL of Minimal Essential Medium (MEM) (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) and penn-strep (Cellgro, Manassas, VA). Cells were seeded at 10^4 cells.cm⁻² and grown to 70% confluence over 48 hours before being harvested with trypsin-EDTA (Cellgro, Manassas, VA). After neutralizing the trypsin with complete medium, the cells were spun down and resuspended in complete culture medium and kept in suspension at 37°C.

Blood was collected from normal donors in 10 mL (EDTA K2/K3) vacutainers (BD, Franklin Lakes, NJ). The blood was washed by centrifugation and resuspended in RPMI medium and then layered onto Histopaque 1077 density medium (Sigma-Aldrich, St Louis, MO). After centrifugation for 600 g x 30 minutes, peripheral blood mononuclear cells (PBMNs) were carefully pipetted from the top of the density medium, washed twice in RPMI, and finally resuspended in RPMI. Erythrocytes were recovered from the pellet fraction and similarly washed and resuspended in RPMI.

Cell morphology was investigated by microscopic evaluation of Wright-Giemsa stained CytoSpin slides, by electrosizing using a CASY counter (Schärfe System, Reutlingen, Germany) and by forward and side scatter using a flow cytometer (CyFlow SL, Partec, Görlitz, Germany).

Primary human tumor cells were obtained in suspension from ascitic fluid derived from routine treatment of a patient with stage 3 c serous ovarian cancer using an IRB approved protocol and with patient consent. Cells from the ascitic fluid were gently pelleted by centrifugation at 600 g x 15 minutes and resuspended in RPMI medium until they were examined by dFFF.

Hollow glass beads

To provide standardized particles of known dielectric and density properties for verifying the dFFF theoretical equations and measuring $P(f)$, hollow glass beads (HGBs) (Cat. 19823-5, Polysciences, Warrington, PA) of different densities and in the size range 3 to 20 microns were fractionated into known densities. This was accomplished by isopycnic separation on a sucrose density gradient prepared by gently layering 12 glucose solutions atop one another in a centrifuge tube (solutions ranged from 50% sucrose by weight [$\rho = 1230$ kg.m⁻³] to 0% sucrose by weight [$\rho = 1000$ kg.m⁻³] in 5% steps). A suspension of

mixed beads was carefully layered on top and the tube was subjected to centrifugation at 900 g x 45 mins. Following centrifugation, bead fractions were carefully withdrawn by pipette from different heights in the centrifuge tube corresponding to the sucrose layers of different densities. The recovered beads were then washed in PBS to remove the sucrose and stored in Eppendorf tubes for later use.

Dielectrophoretic field-flow fractionation (dFFF)

Batch mode dFFF experiments were conducted using a thin, flat horizontal chamber equipped on its floor with an array of parallel, gold electroplated copper microelectrodes on a Kapton substrate as described earlier^{26,27} (for chamber parameters, see Table 1). Sinusoidal electrical signals in the frequency range $15\text{ kHz} < f < 300\text{ kHz}$ at 2.8 V p-p and up to 2 A were applied to the microelectrode array from a custom signal generator. Eluate flow through the chamber at flow rates from 0 – 10 mL.min⁻¹ was provided by a gear pump (Ismatec, Glattbrugg, Switzerland) via a 0.2 µm filter to remove stray particles. Cells leaving the chamber through the exit port were counted and sized by laser light scatter (PC2400D, ChemTrac Systems, Norcross, GA) and data in the form of elution times and cell sizes were collected on a PC using custom software and analyzed with MATLAB scripts.

The normal eluate buffer consisted of an aqueous solution of 9.5% sucrose (S7903, Sigma-Aldrich, St Louis, MO), 0.1 mg.mL⁻¹ dextrose (S73418-1, Fisher, Fair Lawn, NJ), 0.1% pluronic F68 (P1300, Sigma-Aldrich, St Louis, MO), 0.1% bovine serum albumin (A7906, Sigma-Aldrich, St Louis, MO), 1 mM phosphate buffer pH 7.0, 0.1mM CaAcetate, 0.5mM MgAcetate and 100 units.mL⁻¹ catalase (C30, Sigma-Aldrich, St Louis, MO). The eluate was adjusted to a conductivity of 30 mS.m⁻¹ with KCl. For experiments at different osmolarities, the sucrose concentration in the eluate was adjusted between 3.7% (eluate=120 mOs) and 9.5% (eluate=320 mOs).

The total power input to the DFFF chamber at the maximum 2A RMS current and 2.8Vp-p voltage was ~4W distributed evenly over the DFFF chamber area of 0.3 m long x 0.025 m wide, an input power density of only 530 W.m⁻². The chamber area allowed significant passive heat loss under the ambient conditions of ~23°C and the measured temperature increase of the eluate was at most 1.5°C as it moved the length of the chamber from inlet to outlet. This was considered to be sufficiently low not to significantly perturb the cell properties and active heat control was therefore not used during experimentation.

Prior to experimentation, the chamber was flushed with deionized water for 30 minutes and eluate buffer for 6 minutes. Before each dFFF run, the electric field and eluate flow were turned off and 30 µL of cell suspension in RPMI containing $\sim 3 \times 10^4$ cells was mixed with 300 µL eluate buffer and injected into the front end of the chamber via a septum to fill 6% of its length. Injected cells were allowed to settle for 8 minutes (tumor cells) or 10 minutes (PBMNs) before the electric field and eluate flow were turned on to initiate a dFFF run.

Results and Discussion

Density measurements

HGBs are stable dielectric particles exhibiting a large spread of sizes and densities yet a uniform, low polarizability over a wide range of frequencies; effectively, they behave as though $f_0 \rightarrow \infty$ and $f_{CM}(f) \rightarrow 0.5$ in eqn (4). Elution times for HGB fractions of known, narrow density ranges that had been prepared by centrifugation on a sucrose density gradient were measured by dFFF and compared with predictions of Eqns 1 & 2 (see Fig 1A). Once $P(f)$ had been adjusted to allow for loss of local field intensity from the effects of electrode polarization and inductance and resistance in the leads and bus lines, excellent agreement

was found between theory and experiment (Fig 1A). Furthermore, by exploiting the frequency-independent polarizability of the beads, $P(f)$ could be determined experimentally as a function of frequency and used in subsequent dFFF analyses. Beads of the same density but of different sizes exhibited similar elution times, showing that, as expected, particle size did not confound density determinations by the dFFF method. The relationship between elution time and density shown in Fig(1A) was used to determine cell density profiles at 15 kHz in subsequent experiments.

To obtain accurate density measurements directly from elution profiles, the low frequency regime requires that $f \ll f_0$ and $f_{CM}(f) \rightarrow -0.5$ in eqn(4). While there is no theoretical minimum frequency, electrochemical species tend to be generated at DEP electrodes below 10 kHz that can damage cells²⁸. To avoid this, a frequency of 15kHz was chosen for cell density measurements. As a secondary precaution, the eluate contained catalase, which eliminates H_2O_2 , the primary electrochemical byproduct shown in earlier work to affect cell viability when cells were subjected to prolonged exposure to electrodes driven at low frequencies²⁹.

At the eluate conductivity of 30 mS.m^{-1} , crossover frequencies for blood cells were all above 100 kHz and $f_{CM}(15\text{kHz}) \rightarrow -0.49$, satisfying the condition for accurate density measurements. However, crossover frequencies for the tumor cells were found to be around 30 kHz. In this case, $f_{CM}(15\text{kHz}) \rightarrow -0.33$, showing that a correction to the density mapping was required. To achieve this, the density and crossover frequency analyses (see later) were conducted iteratively until consistent results were found.

Using appropriate mappings, cell density profiles (Fig 1C) were derived from dFFF elution profiles (Fig 1B) measured at 15 kHz for erythrocytes, PBMNs, MDA-MB-435, and MDA-MB-231 cells. The distributions found for erythrocytes (peak $\rho_p = 1100 \text{ kg.m}^{-3}$) and PBMNs (peak w/shoulder from $\rho_p = 1055$ to 1075 kg.m^{-3}) match those expected from the literature. Both tumor lines exhibited density distributions centered around $\rho_p = 1060 \text{ kg.m}^{-3}$ that significantly overlapped the PBMN density distribution.

We also used dFFF to follow the dependency of cell densities on osmolarity in the range 120 to 320 mOsm (Fig 1D). A perfect osmometer that follows the Van't Hoff equation is expected to show a linear density dependency on osmolarity with a slope of unity. As expected, erythrocytes behaved more like perfect osmometers (slope=0.69)²⁹, than PBMNs (slope=0.3) and MDA-MB-435 cells (slope=0.44), which are both known to exhibit dynamic volume regulation that help to compensate for changes in osmolarity³⁰.

Hydrodynamic lift

The transport behavior of cells in blood vessels and capillaries is determined by complex interactions that depend on flow rate and vessel diameter and include hydrodynamic forces that distort the cell shape and influence positioning relative to vessel walls^{22,23,31}. By measuring the FFF elution times for cells as a function of flow rate in the absence of DEP forces, the geometry parameter Φ that scales the hydrodynamic lift^{22,23} for deformable particles was measured for erythrocytes, PBMNs and MDA-MB-435 cells. PBMNs were found to have the smallest ($\Phi = 0.04$), erythrocytes an intermediate ($\Phi = 0.08$), and MDA-MB-435 cells the highest ($\Phi = 0.12$) lift parameter at physiological osmolarity. The geometric lift factor increases according to how greatly cells deviate from sphericity under non-shear conditions and/or exhibit greater deformability under shear stress^{22,23}. Because PBMNs and MDA-MB-435 cells relax to spherical morphologies when suspended in the absence of shear stress, the geometry parameters for these cell types reflect their deformabilities. Therefore, the larger geometry factor of the cancer cells indicates that they are much more deformable than PBMNs. Whereas the tumor cells and PBMNs moved

steadily through the dFFF chamber in the hydrodynamic lift experiments (observed through the transparent acrylic top of our chamber using a long working distance microscope), erythrocytes exhibited “tank-treading”³¹ motion in which their position alternated between contact with the chamber floor and hydrodynamic levitation far from it. Given this chaotic behavior, which under our flow conditions is almost certainly accounted for by the erythrocyte’s discoidal morphology³¹, the geometry factor found here for erythrocytes must be considered to an effective value that cannot be compared with the cancer cells and PBMNs.

Total capacitance and surface area

The DEP crossover frequency is the most difficult parameter to derive from dFFF analysis because both sedimentation and hydrodynamic forces are significant when the DEP force passes through zero and changes direction at f_0 . Therefore, both the cell density ρ_p and the hydrodynamic lift geometry parameter Φ need to be taken into account when mapping from cell elution times to crossover frequencies.

We derived DEP crossover frequencies using swept frequency dFFF wherein the applied field frequency was swept downwards during the run according to the logarithmic characteristic shown in Fig 2A. A mapping surface derived from Eqn (7) for dFFF elution profiles in this swept frequency regimen assuming a hydrodynamic lift geometric parameter $\Phi = 0.100$ is shown in Fig 3B. Using this surface, elution times for cells of known density may be mapped to corresponding crossover frequencies. In practice, this mapping was realized by describing the calculated mapping surface in terms of fitted polynomials and then computing crossover frequencies for the cells directly from their elution times.

Because the cell density and hydrodynamic lift values are unknown on a cell-by-cell basis, values for these parameters must be assumed prior to mapping from elution time to crossover frequency and this assumption creates errors in the derived crossover frequency profile. Nevertheless, the spread of densities was found to be small for each cell type examined and the dependency of elution times on the spread of hydrodynamic lift parameters also proved to be small for the wide frequency sweep that was used. Because of this, errors in the derived crossover frequencies caused by assuming mean values for cell density and hydrodynamic lift parameters were small in comparison to the broad spread of crossover frequencies found for the cells.

Typical elution profiles for erythrocytes, PBMNs and MDA-MB-435 and the corresponding crossover frequency profiles are shown in Fig 2C and 2D, respectively. The crossover frequency distribution for PBMNs ranged from below 100 kHz to above 160 kHz with peaks at 110 kHz and 150 kHz that corresponded to the granulocyte and lymphocyte subpopulations of the PBMNs as confirmed by the size distribution of cells observed in the elution profile by the particle detector ($R \sim 3.4 \mu\text{m}$ for lymphocytes, $R \sim 4.0 \mu\text{m}$ for granulocytes). Erythrocytes were found to have an effective crossover frequency of 115 kHz by the dFFF method. Erythrocytes are known to have different crossover frequencies along the directions parallel to and perpendicular to the discoid²⁸. The value observed by dFFF appears to correspond to the lower crossover frequency value, parallel to the disc. The crossover frequencies for MDA-MB-435 cells ranged from 20 to 45 kHz with a mean value of 30 kHz and were found to depend on cell size. For all cell types, the mean observed crossover frequencies agreed well with the values measured by electrorotation and DEP crossover frequencies published earlier (see Table 2), showing that the dFFF method is consistent with other electrokinetic methodologies.

In earlier studies, we showed that as cell volume adapted to imposed changes in the suspending medium osmolarity, the total cell membrane surface area was nevertheless

conserved. This conservation was accommodated by adjustments in cell surface morphological features including folds and microvilli. To verify that our dFFF analysis provided data consistent with this finding, we undertook measurements of DEP crossover frequencies versus osmolarity (Fig 3A). Eqns (5) allow this data to be interpreted in terms of total membrane capacitance C_{tot} , a measure of the cell total membrane area. It is clear from the independence of the derived C_{tot} values on osmolarity (Fig 3B) that our results are consistent with our earlier studies showing conservation of membrane area. Assuming that cell plasma membrane has a specific capacitance of $8\text{--}9\text{ mF.m}^{-2}$ when flat³⁸, then the mean membrane areas found from Fig 3B for erythrocytes, PBMNs and MDA-MB-435 cells, respectively, are $1.8 \times 10^{-10}\text{ m}^2$, $3 \times 10^{-10}\text{ m}^2$, and $2 \times 10^{-9}\text{ m}^2$.

Many cancers are heterogeneous and exhibit a much wider range of cell sizes than normal tissues. Furthermore, the cytoplasmic to nuclear ratio (CNR) of tumor cells tends to vary among differently sized subpopulations. Because the density of the nucleus is usually higher³⁸ (typically $>1200\text{ kg.m}^{-3}$) than the cytoplasm (typically 1020 kg.m^{-3}), cells having different CNRs are expected to show corresponding density differences. For MDA-MB-435 and MDA-MB-231 cells, the CNR was found to be greater in large cells than in smaller ones (see later). In Fig 1C, individual density profiles for MDA-MB-435 from $20\text{ }\mu\text{m}$ to $7\text{ }\mu\text{m}$ in diameter are shown overlaid (though they are not individually labeled because they are so similar). This similarity shows that, in these cancer lines, cell density is independent of cell size, showing that the density of the cell nuclei must be similar to that of the cytoplasm. This finding is consistent with the observation that nuclear material is generally much less tightly organized in tumor, compared with normal, cells³⁹.

Dependency of cell surface area on cell radius

Data is shown in Fig 3C for mean C_{tot} plotted against mean R for 16 cell types for which we have published DEP data previously, including erythrocytes, the 6 main subtypes of PBMNs and 9 different cultured cell lines (see Table 2). The slope of the plot shows that C_{tot} is proportional to $R^{3.01}$ for these 16 cell types, indicating that a direct relationship exists between cell surface area and cell volume. This proportionality seems inconsistent with allometric scaling laws that describe the relationships between surface area, mass and metabolic rate of living organisms over many orders of magnitude of size range³⁴. However, these laws are considered to reflect scale-dependent nutrient and heat transport processes that ultimately involve diffusion limitations⁴¹. The cell types in Fig 3C came either from human blood or from tissue culture, both nutrient-rich, temperature-regulated environments where diffusion is not limiting for cell size or metabolic rate. Therefore, the size and metabolism of cells under these conditions must be controlled by intrinsic cellular mechanisms. Adaptation of cell membrane area to changing metabolic activity is well established^{42,43}, and under these non-limiting conditions, it seems reasonable that, cells would adjust their membrane surface areas to accommodate the transmembrane transport needed to support their function and metabolism. Because these processes occur on a cell volume basis, cell membrane surface area would therefore be expected to be proportional to cell volume, as we observe.

dFFF analyses revealed that MDA-MB-435 suspensions exhibited a wide range of differently sized cells having different C_{tot} values. Significantly, a plot of C_{tot} against R for the different cell sizes within MDA-MB-435 suspensions (Fig 3C) followed a $R^{1.24}$ dependency. This power is far smaller than predicted by either allometric scaling or volume metabolic considerations, indicating that yet another physical principle must control membrane areas among the MDA-MB-435 size subpopulations under our suspension conditions. While investigating this problem, we were surprised to discover that the distribution of sizes within the suspensions of these and other adherently-cultured cells was

not stable but changed with time. Specifically, following harvesting and suspension in complete media, the proportion of large MDA-MB-435 cells diminished with time while the proportion of small cells increased (Fig 3D). As these relative proportions altered, the dependency of C_{tot} on $R^{1.24}$ was maintained for the cell size subpopulations.

Microscopic examination showed that the changing size distribution within the cancer cell suspensions resulted from cell cytoplasmic shedding (Fig 4). Immediately after initial suspension in complete media, the cancer cells exhibited a ragged periphery with lamellopodia and blebs on their surfaces (Fig 4A). Over the course of several hours, these features were gradually pinched off and floated away into the suspension as a shower of vesicles ranging from $< 1\mu\text{m}$ to $\sim 3\mu\text{m}$ in diameter. As a result of this ongoing process, cells lost both membrane and cytoplasm and became smaller with time (Fig 4B). This phenomenon, illustrated in Fig 4C, created cells exhibiting a range of different morphologies having widely differing cytoplasmic to nuclear ratios (Fig 4D). Cells in the initial stages of cytoplasmic loss maintained viability as judged by trypan blue dye exclusion and could still be grown in culture. However, once cytoplasmic loss had progressed so that cells resembled nuclei with very small cytoplasmic margins, they could no longer be cultured and died.

Although some cells did show the characteristic morphology of apoptotic bodies, the cytoplasmic loss mechanism seemed to be distinct from apoptosis⁸ and anoikis⁹, which do not normally halt once initiated, and it did not resemble necrosis in which membrane barrier function is rapidly lost. We found that the cytoplasmic loss could be inhibited by adding 10 mM Blebbistatin to cells during and after harvest. This agent inhibits non-muscle myosin⁴⁴ (NMM) that plays a role in several membrane biomechanical activities⁴⁵ including blebbing and the formation of lamellipodia and its inhibiting action suggested that active physiological processes within the cell membrane were involved in extruding and pinching off the cytoplasm. If tension within the membrane produced by NMM drives the shedding then, assuming cell membrane partitions between the cell and the shed vesicle in relationship to tension, the membrane area that remained with the cell would tend to be proportional to the cell radius, leading to a $C_{tot} \propto R$ relationship. This is close to the $R^{1.24}$ dependency observed for the MDA-MB-435 suspensions (Fig 3C) and suggests that the majority of smaller cells in the suspensions may have been produced either during or after harvest through cytoplasmic loss from initially larger cells. In that case, an important inference is that the distribution of cell sizes observed within harvested cell suspensions may not reflect the distribution of cell morphologies that were present during attached, tumor cell growth.

Implications for circulating tumor cells

Our results have a number of implications for CTCs with respect to their morphology and fate as well as for the efficiency of their isolation from blood by DEP methods. Rapid profiling of cell densities by dFFF showed (Fig 1C) that MDA-MB-435 cells have densities that overlap with PBMNs. Additionally, we have now examined almost 30 different cultured cell lines, ascites and leukemias, and find that, in most cases, density gradient methods could isolate, at best, a small subset of those cells from blood (data not shown). This finding suggests that density isolation methods are, in general, not suitable for CTC isolation applications.

DEP requires that the cell membrane be of low conductivity (and therefore intact) to allow the isolation of cancer cells from PBMNs. Therefore, we were concerned that lysis, if used as a preliminary step to eliminate erythrocytes from patient specimens, might confound the DEP collection of CTCs. However, our osmotic response data indicated that cancer cells can adjust to osmotic stress well, leaving them undamaged and still responsive to DEP. This suggests that lysis can be used for erythrocyte elimination from clinical blood specimens

without affecting subsequent CTC isolation by DEP. Indeed, in a pilot study we used 2% Tris- 0.83% ammonium chloride to reduce the number of red blood cells in ovarian ascitic fluid prior to dFFF isolation (data not shown). This reduced the red blood cell count but did not affect the number of viable tumor cells.

The hydrodynamic lift properties of the tumor cells show that they would experience greater lift away from blood vessel walls than lymphocytes, tending to keep them from contacting vessel walls in the circulatory system until they entered capillaries smaller than their own diameter. Even then, the greater deformability of the cancer cells (indicated by their large hydrodynamic geometry parameter) might assist their passage through smaller blood vessels until their large size presented a barrier to further migration. The ability of CTCs to deform has been linked to cytoskeletal deformability that allows their dissemination to metastatic sites. Our results suggest that tumor cells might also reduce their size through cytoplasmic shedding until they could pass through the smallest vessels that PBMNs can negotiate. Together, these effects may help account for how CTCs manage to negotiate the capillary beds of the circulatory system and suggests that initial tumor cell size is less of a barrier to tissue infiltration than might have been expected.

Interestingly, loss of cytoplasm also implies potential loss of cell type memory because a significant part of that memory lies within the cytoplasm⁴⁶. Indeed, cytoplasmic loss tends to be a characteristic of cell dedifferentiation⁴⁷. Therefore, the smallest cell shown in Fig 4D, which had lost as much as 97% of its initial cytoplasm, potentially had lost most of its memory and could show a strongly modified gene expression profile. Several laboratories have compared the gene or proteomic signature of paired primary tumor-metastatic tissues⁴. While in some cases little or no differences were found, in other cases the differences were vast⁴. Ultimately, the farthest CTCs might be able to migrate and engraft into peripheral tissue and the extent to which their gene expression might be modified could depend on many different factors including the minimum size at which CTCs maintain viability after shedding cytoplasm.

In addition to MDA-MB-435 and MDA-MB-231, we have examined cells from many other adherent cell types following harvest. In every case we found evidence for some degree of cytoplasmic shedding, even during routine passage of the cultures, until the cells reattached to a growth surface. The processes that lead to this shedding are unclear, but cellular shedding is known to be one way of protein secretion into the extracellular matrix⁴⁸.

While a wide range of morphologies are evident among CTCs collected from clinical specimens^{49,50}, it is not feasible to study the morphology of CTCs as a function of time because they are so rare. However, it is possible to examine human cancer cells that have been shed from primary tumors into the peritoneum and have accumulated in ascitic fluid. While these cells have not been exposed to the same conditions that CTCs experience in the circulatory system, they nevertheless provide a plentiful and realistic *in vivo* source of shed cancer cells for morphological examination. Fig 5 shows the appearance of Wright-Giemsa stained ascitic cells from an ovarian cancer patient. The tumor cells are larger and have irregular nuclei compared with the smaller, nucleated lymphocytes and the erythrocytes in the preparation. While the lymphocytes and erythrocytes have essentially uniform characteristic diameters, the tumor cells exhibit a wide range of sizes and cytoplasmic to nuclear ratios. Furthermore, the peripheries of the cancer cells show evidence of lamellipodia and blebbing that are consistent with the cytoplasmic shedding events seen in our harvested cells (Fig 5). Therefore, it appears highly likely that cell size and cytoplasmic to nuclear ratio are also dynamic entities in these patient-derived ascites tumor cells. This result lends support to the concept that the range of CTC morphologies observed in clinical specimens^{49,50} also derives, at least to some extent, from cytoplasmic loss processes.

Microvesicles⁵², or microparticles, are self-contained droplets of cytoplasm surrounded by cell plasma membrane that are generally considered to be cell fragments. They occur in the circulation and tend to be particularly abundant in the blood of cancer patients^{52,53}. Tumor-derived microvesicles are believed to play a significant role in thrombosis-related complications⁵³, inflammation, cancer cell invasion⁵⁴, modulation of the immune system that can reduce the body's resistance to cancer⁵⁵ and the transfer of gene-modulating signals that may enable cancer progression⁵¹. Our data suggest that tumor cell detachment from the growth site and the shedding of cytoplasm in the form of abundant microvesicles that range in size up to several microns in diameter go hand in hand with one another. Therefore, the presence of tumor-derived microvesicles and CTCs in the blood may be highly correlated.

DEP isolation of circulating tumor cells

Most current methods for isolating CTCs utilize a cell surface marker such as EpCAM to capture and concentrate the target cells^{55–58} but this marker is absent from approximately 15% of solid tumors, most soft tissue tumors and all lymphomas, and from CTCs that are progenitor-like and may be of enhanced risk for initiating metastasis^{59,60}. Size-based filtering^{61,62} offers a surface-marker-free isolation approach for CTCs but this method captures only tumor cells that are larger than peripheral blood mononuclear cells. DEP^{10–13} offers an alternative surface-marker-free isolation approach to rare cell isolation^{14–16} that discriminates between cells on the basis of their total membrane capacitance, which is proportional to their cell membrane surface area. This parameter increases both with increased cell size and increased membrane-rich features (such as folds and microvilli) that are more prevalent in highly metabolizing cells^{42,43}, such as cancer, than in peripheral blood cells. Unlike most marker-based counting methods, DEP allows viable cells to be isolated for extensive molecular analysis and possible culture.²⁶

CTCs typically occur at a concentration of a few cells per 10 mL of peripheral blood and their detection demands the processing of up to 10⁸ peripheral blood mononuclear cells on a timescale of 1 to 3 hours⁴. We showed many years ago that DEP could be used to isolate tumor cells from blood on small microelectrode arrays^{14,15}. To achieve sufficiently high throughput for clinical applications, however, we^{16,17} and Markx¹⁸ introduced macroscopic field-flow-fractionation (FFF) principles to provide the method of dFFF, which we have applied to rare cell isolation²⁶. Because cells must remain spaced by several diameters to avoid dipole-dipole interactions that can perturb their normal DEP responses²⁶, the loading capacity of this method is limited to a few million cells per run and many batches need to be processed to achieve CTC analysis of 10 mL blood. However, we recently developed a variant of dFFF in which the cell mixture is injected continuously from an inlet slit in the chamber bottom with eluate flowing above it, the cells reach equilibrium heights as they flow across the DEP electrode in the chamber, and the CTCs are skimmed from the bottom through an exit slit while blood cells flow to waste. This continuous method can process more than 10⁶ cells.min⁻¹, allowing a 10 mL blood specimen to be processed in about 45 minutes as long as the relevant differences between cancer cell types, the CTCs they spawn, and the blood cells from which they must be isolated are known. The analytical methods described in this article provide the needed understanding of these parameters.

The total cell capacitance for all cancer cell types that we have examined greatly exceeds that of blood cell types (some of these are shown in Fig 3C) indicating that they are all amenable to isolation from blood by DEP. Significantly, this pertains even for the MDA-MB-435 (Fig 3C) and MDA-MB-231 (data not shown) cells that had shed cytoplasm to the extent that they had become as small as PBMNs. This finding is significant because it shows that DEP can capture viable cancer cells even if they are too small for isolation by mechanical filtering^{61,62} methods.

Conclusions

Metastatic disease is considered to be the greatest threat to patient survival in the many cases for which effective treatments for primary tumors have been developed. Considerable effort is being focused on isolating the circulating tumor cells and understanding how they shed, survive, migrate and engraft at distal sites to form metastases. Little is known about how the form and function of cancer cells change after they detach from their tumor of origin and undertake the journey as CTCs in the challenging environment of the circulatory system. dFFF provides the means not only to isolate CTCs from blood but also to characterize and profile cancer cells derived from solid tumors according to their membrane capacitance, total surface area, density, and hydrodynamic properties. Unfortunately, it is not feasible to study these properties of CTCs as a function of time or in a statistically meaningful way using clinical specimens because CTCs are so very rare (typically less than 1 CTC per mL of whole blood) and take so long to isolate (> 1 hour). Therefore, we have necessarily focused on the time-dependent changes of cultured cells as they are released from their growth matrix and built a largely circumstantial case for similar phenomena occurring when cancer cells leave tumors and become CTCs *in vivo*.

Our results show that tumor cells are not, in general, likely to be amenable to isolation from PBMs by density-based methods. The cell lines we examined exhibit dynamic volume responses that suggest they can endure osmotic challenges that would allow cell lysis to be used to eliminate erythrocytes from clinical blood specimens for CTC preparation purposes. Tumor cells exhibited significantly higher hydrodynamic lift than blood cell types, suggesting that they would tend to stay away from blood vessel walls in the circulatory system until they reach capillaries having comparable diameters to the cells themselves. Even then, our data show that they have considerable deformability that might facilitate their passage through smaller vessels than their diameters might suggest.

Our results also suggest that the detachment of cancer cells from solid tumors may lead to significant time-dependent structural modifications that impact observed cell morphology, including size, membrane area and nuclear to cytoplasmic ratio. Indeed, they are consistent with the hypothesis that the wide range of morphologies observed for CTCs within single clinical specimens reflects the consequences of cells that perhaps initially possessed similar morphology but subsequently underwent different degrees of cytoplasmic shedding following their detachment from the primary tumor. Our tissue culture data for tumor cells suggested that, after harvest, the process of cytoplasmic loss is typical and is ongoing until cells reattach to a suitable substrate and resume growth. If cells failed to reattach soon enough in our experiments, then many lost so much cytoplasm that they eventually died. Interestingly, the death process seemed to be distinct from conventional apoptosis, anoikis and necrosis.

Finally, despite shedding cytoplasm and falling in size, the total membrane capacitance of cancer cells remained higher than that of PBMs of similar size, showing that DEP should be able to capture CTCs even when they are relatively small. Nevertheless, DEP depends upon membrane integrity for the dielectric differences it exploits for cell discrimination and isolation. While this means it is not applicable for isolating necrotic CTCs or CTC debris, it offers the advantage that viable CTCs may be isolated for detailed molecular analysis and for potential culture for drug sensitivity tests. Furthermore, the DEP method offers the significant advantage that it does not depend on cell surface markers, which are not expressed by many cancers or by cancer progenitor cells.

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Fig. 1.

(A) Dependency of the dFFF elution time for hollow glass beads as a function of their density difference compared to the eluate buffer. — — theoretical relationship based on Eqns 2 and 3 scaled by the best fit of the voltage loss parameter $P(f)$ (see text). (B) Elution profile for MDA-MB-435 cells in an eluate of 250 mOsm at a DEP frequency of 15 kHz. (C) Cell density distribution profiles for an eluate of 250 mOsm derived by using the dependency in A as a mapping for elution profiles like those shown in B. (D) Variation of cell density, derived from dFFF, as a function of eluate osmolarity. (Red: erythrocytes; Black: PBMNs; Blue: MDA-MB-435 for all panels).

Fig. 2.

(A) Time dependency of the applied DEP field frequency used in the swept-frequency dFFF regime (see text). (B) Theoretically derived mapping between cell elution times in swept regime dFFF and cell DEP crossover frequency for an assumed hydrodynamic lift geometry factor of 0.1. (C) Swept-frequency regime dFFF elution profiles for the cell types at 250 mOsm. (D) Distributions of cell DEP crossover frequencies derived from elution profiles in C using a polynomial fit to the mapping shown in B. (Red: erythrocytes; Black: PBMs; Blue: MDA-MB-435 for all panels).

Fig. 3.

(A) Variation of the cell DEP crossover frequencies with eluate osmolarity. (B) The cell total cell capacitance values, derived from A using Eqns 5, is maintained constant as the osmolarity is altered through changes in cell surface folding. (C) Cell total capacitance plotted as a function of size for 16 different cell types shown in Table 2. The diamonds show different blood cell types and the squares are tumor cell lines. The blue filled circles show subpopulations having different sizes within a suspension of MDA-MB-435 cells. (D) Time dependency of cell size ranges within a suspension of MDA-MB-435 cells. Initially, most cells are in the 11 to 18 μm size range but, as time passes, shedding of cytoplasm in vesicles leads to increasing proportions of smaller cells.

Fig. 4. (A) Wright-Giemsa stained slide showing the morphology of MDA-MB-435 cells immediately after harvest. Cell peripheries are rich in lamellipodia and blebs and several protrusions of the cytoplasm may be seen detaching. (B) After remaining in a suspension in complete tissue culture medium for 2 hours, the morphology of MDA-MB-435 cells shows the effects of continued shedding of cytoplasm. Although most microvesicles are lost during the cytocentrifuge slide-making process, a few may be observed between the cells in this field. (C) The cytoplasmic shedding results in loss of cytoplasm and membrane from the cells. (D) Images of cells illustrating the range of cell morphologies found in MDA-MB-435 suspensions following two hours in suspension. The cytoplasmic to nuclear ratio (CNR) cover a wide range as they do for circulating tumor cells found in clinical specimens.

Fig. 5. Wright-Giemsa stained slide showing human ovarian cancer cells (large with complex nuclei) obtained from ascitic fluid. Cell peripheries show signs of blebbing and protrusions and a range of morphologies having widely different cytoplasmic to nuclear ratios is evident just as in the case of cultured cells that have stood in suspension for several hours.

Table 1

Definitions of parameters in the equations used to derive the biophysical properties of cells from measured dFFF elution characteristics.

Symbol	Parameter	Value	Units
F_{sed}	Sedimentation force on cell in eluate	*	N
F_{DEP}	Dielectrophoretic force on cell in eluate	*	N
F_{HDL}	Hydrodynamic lift force on cell in eluate	*	N
ρ_p	Cell Density	*	kg.m ⁻³
h	Equilibrium height of cell above DEP array	*	m
f_0	Cell crossover frequency	*	Hz
Θ_0	Cell crossover frequency per unit eluate conductivity = f_0 / σ_s	*	Hz.S ⁻¹
$\Phi(v)$	Cell deformability factor	*	—
C_{mem}	Cell specific membrane capacitance	*	F.m ⁻²
C_{tot}	Cell total membranecapacitance	*	F
R	Cell radius	measured	m
V	Applied DEP AC voltage	2.8	Volts pk-pk
f	Applied DEP AC frequency	See methods	Hz
ρ_s	Density of eluate	$1.036 \times 10^3 \text{ }^\dagger$	kg.m ⁻³
σ_s	Conductivity of eluate	$3.0 \times 10^{-2} \text{ }^\dagger$	S.m ⁻¹
$\epsilon_s \epsilon_0$	Dielectric permittivity of eluate	$6.90 \times 10^{-10} \text{ }^\dagger$	F.m ⁻¹
η	Kinematic viscosity of eluate	$1.266 \times 10^{-7} \text{ }^\dagger$	m ² .s ⁻¹
B	Eluate flow rate	$(3 - 15) \times 10^{-8}$	m ³ .s ⁻¹
s	Microelectrode width and spacing	5.0×10^{-5}	m
d	Microelectrode periodicity = $4s$	2.0×10^{-4}	m
H	DEP chamber height	3.62×10^{-4}	m
W	DEP chamber width	2.4×10^{-2}	m
L	DEP chamber Length	0.30	m
$P(f)$	Effective proportion of DEP voltage	0.7	—
$a = \frac{4}{3} \pi g$	$\frac{4}{3} \pi$ x acceleration due to gravity	41.08	m.s ⁻²
$b = 352 \pi \epsilon_s \epsilon_0 d^{-3}$	Constant for this study	9.54×10^4	F. m ⁻⁴
$c = \frac{6\eta}{H^2 W}$	Constant for this study	241.5	m ⁻¹ .s ⁻¹

* parameter value deduced from analysis of dFFF elution measurements

[†]The values given pertain to the eluate used in this study comprising an aqueous solution of 9.5% sucrose adjusted to a conductivity of 30 mS.m⁻¹ (see methods)

Table 2

Total capacitance data for cell types plotted in Fig 3C

Cell type	Index in Fig 3C	Radius, r, μm	$C_{tot}^{\dagger}$, pF	Reference
Erythrocytes	a	2.8	1.142835	32
B-Lymphocytes	b	3.4	1.439597	33,34
T-Lymphocytes	c	3.4	1.9306	33,34
Basophils	d	3.58	1.803822	33
Neutrophils	e	4.06	2.038246	33
Eosinophils	f	4.19	2.071587	33
Monocytes	g	4.21	3.169411	33,34
DS19 HMBA	h	5.2	5.198854	35
DS19 unTX	i	5.5	6.614304	35
HL-60	j	5.8	7.165313	36
MDA- 231	k	6.2	12.37093	14
6M2 non-perm	L	6.4	15.59596	37
MDA- 468	m	7.2	18.50741	Unpub
HT-29AS15	n	7.62	11.45563	Unpub
HT-29	o	7.75	14.64248	Unpub
MDA-435	p	7.7	22.32943	This work

[†] Cell total capacitance values were calculated from literature values of cell crossover frequencies, f_0 , or membrane specific capacitance, C_{mem} , using Eqn5 5.