Automated electroration shows electrokinetic separation of pancreatic cancer cells is robust to acquired chemotherapy resistance, serum starvation, and EMT

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We used automated electroration to measure the cytoplasmic permittivity, cytoplasmic conductivity, and specific membrane capacitance of pancreatic cancer cells under environmental perturbation to evaluate the effects of serum starvation, epithelial-to-mesenchymal transition, and evolution of chemotherapy resistance which may be associated with the development and dissemination of cancer. First, we compared gemcitabine-resistant BxPC3 subclones with gemcitabine-naïve parental cells. Second, we serum-starved BxPC3 and PANC-1 cells and compared them to untreated counterparts. Third, we induced the epithelial-to-mesenchymal transition in PANC-1 cells and compared them to untreated PANC-1 cells. We also measured the electroration spectra of white blood cells isolated from a healthy donor. The properties from fit electroration spectra were used to compute dielectrophoresis (DEP) spectra and crossover frequencies. For all three experiments, the median crossover frequency for both treated and untreated pancreatic cancer cells remained significantly lower than the median crossover frequency for white blood cells. The robustness of the crossover frequency to these treatments indicates that DEP is a promising technique for enhancing capture of circulating cancer cells. *Published by AIP Publishing.* [<http://dx.doi.org/10.1063/1.4964929>]

I. INTRODUCTION

Cancer is a leading cause of death, a statistic that is made worse by many cases of late detection, often after the onset of metastasis and its characteristic spread of cancerous cells away from the primary tumor to form new tumors throughout the body. An expected mediator in this process is circulating tumor cells (CTCs), which are cancer cells found in patient blood at an extremely low concentration, about 1–10 CTCs per ml of blood.¹ These cells originate from tumors and a subpopulation has been shown to be pre-cursors to metastasis.² In addition to this putative role, it is well known that these circulating tumor cells correlate with disease progression,³ that they can be captured even in early disease,⁴ and that they can be used to gauge functional cell responses to pharmaceuticals⁵ or the genetic character of the cancer.⁶ This information from captured CTCs is valuable to both clinicians and researchers particularly in the context of pancreatic cancer, in which the presence of circulating pancreatic cells precedes

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tumor formation.² Furthermore, circulating pancreatic cells exist in a subset of patients without cancer but with potentially cancerous pancreatic cystic lesions.⁴

To obtain this information, CTCs have been successfully captured from blood samples through mechanical,⁷ electromechanical⁸ and immunoaffinity methods,^{9–11} the last being the most common. However, challenges exist capturing subsets of CTCs that have reduced antigen expression, often associated with the epithelial to mesenchymal transition (EMT)¹² or acquired resistance to chemotherapy.¹³ Also, performing more complex and informative procedures requires a higher purity of captured cells, and improvements to the current state-of-the-art are needed.

This improvement could be achieved through dielectrophoresis (DEP). The use of non-uniform alternating current electric fields can be used to attract and repel objects with different complex permittivity values depending on the field frequency. Using DEP as the only separation mechanism has led to some success in CTC enrichment.^{14–16} A proof-of-concept hybrid DEP-immunoaffinity device has shown promise of further improving capture.^{17–19} Careful device design and frequency selection can attract CTCs while repelling contaminating white blood cells, and simulations have predicted capture efficiency increases of up to 400%.²⁰

The upper half of Figure 1 shows measured DEP spectra for different white blood cells and cancer cell lines. Differences in the crossover frequency, the frequency in which a cell changes from exhibiting negative to positive DEP, between cancer cells and white blood cells permit positive selection of the rare cancer cells within a blood sample. This can be done by operating DEP at an optimal frequency between the two different crossover frequencies.

Unfortunately, measuring DEP forces against frequency in order to measure accurate DEP spectra can be experimentally difficult, because, in order to estimate DEP forces, one must carefully model the electric field intensity and the hydrodynamics of cells as the two effects vary spatially in the device. Furthermore, the magnitude of negative DEP is difficult to measure

FIG. 1. Calculated DEP and ROT Spectra of untreated PANC-1 and BxPC3 pancreatic cancer cells compared to that of U937 leukemia blood cells and white-blood cells from healthy donors. Curves were generated by taking the median of the fit cell electrical properties (specific membrane capacitance [mF/m^2], radius [μm], cytoplasmic conductivity [mS/m], and cytoplasmic relative permittivity [ϵ]) of the corresponding populations: white blood cell: (9.8, 5.6, 721, 111), U937: (14, 7.5, 750, 106), PANC1: (20.2, 12.1, 476, 90), BxPC3: (22.5, 8.1, 453, 91). Cell properties are shown in detail in Table I and Figs. 6–8. Medium of conductivity was measured to be 70 mS/m, and relative permittivity was calculated based on Ref. 31 to be 74.12. Differences in the crossover frequency between the cancer cells and the white blood cells allow for a positive DEP selection.^{15,20,54}

because cells are forced to regions in the device where the field is weak, so DEP trapping experiments yield only partial spectra.²¹ Additionally, near the crossover frequency, which is of greatest interest for cell separations, the DEP signal is necessarily weakest, making it more vulnerable to confounding effects.

A related technique, electrorotation (ROT), relates to DEP forces through the Clausius-Mossotti factor.^{22–24} In ROT, a nearly uniform rotating electric field induces constant torques on (and thus steady rotation rates of) the cells. By measuring cell rotation rate as a function of applied field frequency, one may trace out a ROT spectrum. This spectrum is proportional to the imaginary part of the cell's Clausius-Mossotti factor, which contains all of the information necessary to reconstruct the real part through either the Kramers–Krönig relations or by fitting cells' dielectric properties to a multi-shell model.^{22–25} Corresponding DEP-ROT plots are shown in Figure 1.

Using cell lines exclusively cannot be a direct substitute for capture models for CTCs in the bloodstream because of possible changes in phenotype in these cells. Changes in CTC size, surface markers, and nuclear-to-cytoplasm ratio can occur due to vesicle shedding processes,²⁶ the epithelial-to-mesenchymal transition (EMT)^{9,11,27} in which tumor cells can be reprogrammed as cancer develops, or developed cancer therapy resistance.²⁸ Cell lines have been shown to be too large and express excessive amounts of surface markers^{29,30} relative to bloodstream-captured CTCs. Such acquired differences can also result in different DEP spectra, inducing shifts in the crossover frequency.

Thus, an investigation into these different variants of developmental differences is necessary in order to gain an understanding of the whole picture behind the characterization of CTC electrical properties. In this study, we measured the electrorotation spectra of gemcitabine-resistant, serum starved, and EMT-induced pancreatic cancer cell lines in addition to the parent/untreated cells and healthy donor white blood cells in order to fit the cell electrical properties and compute the full effects on DEP spectra. These measurements can then inform optimal settings for DEP-enhanced cell capture devices.

II. MATERIALS AND METHODS

A. Cell culture and preparation

A low-conductivity isotonic sugar buffer used as the medium for electrorotation was prepared with 9.5% sucrose, 0.3% dextrose, 0.1% Pluronic F68, 0.1% bovine serum albumin (BSA) purchased from Sigma-Aldrich (St. Louis, MO) in de-ionized water, with phosphate-buffered saline (PBS) added until the conductivity was 70 mS/m (with automated temperature compensation to 20 °C), approximately 2 ml of PBS per 50 ml of buffer. This ROT buffer was refrigerated at 4 °C and warmed to 37 °C before experimentation. Modeled as a pure sucrose solution, the media was assumed to have a relative permittivity of 74.2.³¹

For experimentation, 70% confluent adhesion cultures and suspension cultures kept at 3×10^5 to 2×10^6 cells per ml were cultured in Corning CellBIND surface 25 cm² flasks from ThermoFisher Scientific (Waltham, MA) with 5 ml of cell culture media supplemented 10% with fetal bovine serum and 100 units penicillin, 0.10 mg streptomycin, and 0.25 g amphotericin B per ml in a 5% CO₂, 37 °C humidified incubator. Cell cultures included BxPC3 pancreatic, PANC-1 pancreatic, and suspension U937 lymphoma human cancer cells from the American Type Culture Collection (ATCC; Manassas, VA). BxPC3 and U937 cells were cultured with Roswell Park Memorial Institute-1640 (RPMI) purchased from Lonza (Basel, Switzerland), while PANC-1 cells were cultured from Dulbecco's Modified Eagle Medium (DMEM) media purchased from Mediatech (Manassas, VA).

Gemcitabine-resistant subclones were isolated by exposing gemcitabine-sensitive parental BxPC-3 cells to increasing concentrations of gemcitabine over a 10-month period. Clones were isolated at 80 nM and 360 nM gemcitabine, referred to as BxGR-80C and BxGR-360C respectively. Resistance was confirmed by measuring and comparing gemcitabine IC₅₀ values.

For the serum starvation studies, adhesion cell cultures of PANC-1 or BxPC3 cell lines were seeded into two CellBIND flasks 48 h before performing electrorotation studies. One flask

would be passaged with regular media with both the 10% serum and 1% antibiotics, while the other flask was passaged using media with only the 1% antibiotics. We would then perform an electrorotation experiment on both samples, one from each flask.

EMT induction was performed by plating PANC-1 cells and controls at about 6.25×10^3 cells per square centimeter in 6-well plates approximately six days before the scheduled experiment. We then treated the cultures with 10 ng/ml TGF-beta and 20 ng/ml epidermal growth factor (EGF) in reduced serum conditions (2.5% FBS RPMI-1640) on days 1, 3, and 5 before performing electrorotation experiments on the sixth day on both the EMT induced cells and the control cells.

Adhesion cultures were rinsed with PBS twice, and then lifted off by treating in trypsin for 5 min. The suspension was washed three times by centrifuging at $300\times g$ for 5 min, removing the supernatant with a pipette, and re-suspending the cell pellet in 1 ml ROT buffer. Optionally, the cell suspension was then diluted with additional ROT buffer to reduce the number of cells in the field of view to approximately 15.

B. Experimental apparatus

An electrorotation cell with hyperbolic 4-electrode geometry³² was fabricated with Chromium-Gold-Chromium electrodes on 0.5 mm-thick borofloat glass wafers in the Cornell Nanoscale Science and Technology Facility (Ithaca, NY) using standard photolithographic techniques (described in detail in Ref. 18). The tip-to-tip distance between opposite electrodes was $800\text{ }\mu\text{m}$ (similar to Ref. 33), and the hyperbolae extended until the narrowing gap between adjacent electrodes was $133\text{ }\mu\text{m}$ (Figure 2). This gap was sufficiently large such that the device had large impedance compared to the $50\text{ }\Omega$ terminating resistors in parallel with each electrode connection. A 5 mm-diameter plastic well was epoxied around the electrode gap to hold the cell suspension. Four sinusoidal voltages in quadrature (all $3.8\text{ V}_{\text{p-p}}$) were applied to equal length $50\text{ }\Omega$ coaxial cables via a four-channel function generator (ArbStudio 1104, Teledyne Lecroy).

C. Capturing ROT videos

To conduct an electrorotation run, cell suspension ($30\text{ }\mu\text{l}$) was pipetted into the well, and cells settled for approximately 30 s. During the settling period, the function generator applied

FIG. 2. Schematic of the overall ROT device and operation, with plotted sinusoidal voltages and frequencies as shown. (a) A $10\times$ magnified view of the electrodes in the ROT device, with an electric field direction is shown corresponding to the voltages at the marked time frame shown in C. (b) Overhead view of the ROT device, with electrodes silver epoxied to red wires in order to connect to mutually grounded BNC breakout connectors. (c) Sinusoidal voltage functions within each 5 s interval, supplied by the function generator to the separate connectors, each with a different 90° delay from the last one in order to generate an electric field in the direction shown. This configuration would result in a counterclockwise rotating electric field vector. (d) Shuffled frequency 5 s intervals plotted over the entire course of a single ROT experiment. These were randomized in order to eliminate any possible dependence of experimental time on rotation rate (e.g., Joule heating or drifting of cell position).

51.4 kHz ROT signal continuously to prevent the settled cells from adhering to the glass and to reach a temperature steady-state before the start of the ROT data acquisition. We positioned the microscope stage so the field of view was in the approximate center of the electrodes where the field is uniform.³⁴ For an electrorotation run, we programmed 5 s intervals of 36 different frequencies equally spaced on the log scale over the range 1.125 kHz–62.5 kHz into the ArbStudio software, with randomized order to de-correlate the applied frequency with any time-varying effects on the experiment (e.g., drift of cells into varying-strength field, electrical properties varying with temperature, etc.) (Figure 2). This sequence was preceded by a brief pause, then 5 s of clockwise (CW) field, and then 5 s of counterclockwise (CCW) field (both at 51.4 kHz) in order to synchronize the video with a clearly defined pattern in the applied voltage sequence.

We illuminated the cells under brightfield with the condenser aperture wide open in order to minimize exposure times to maximize the frame rate. An Eclipse TE2000U inverted microscope by Nikon (Melville, NY) equipped with a 40×/0.6 Plan Fluor objective, a RETIGA EXi FAST (8-bit images, 1392 × 1040 pixels, no binning), and NIS Elements D software captured a time-lapse of images. We prescribed frame rates at 10 frames per second (FPS), but the camera readout speed limited actual acquisition to ~7 FPS. After each video was captured, we rinsed the well on the ROT device at least three times with deionized water before proceeding with a new sample.

D. Acquisition of individual cell rotation rates from videos

NIS Elements software exported video frames were exported to.tif files, which MATLAB imported for further processing. Manually estimating the rotation rate of the cells is quite tedious, so we developed an algorithm, similar to some found in the literature,^{35–37} to automate parts of the rotation rate estimation.

First, for each pixel spatial location in the video, the script subtracted the background by computing the temporal median brightness and subtracting it from each video frame to suppress debris in the microscope optics and in the ROT chamber and non-uniformity in the illumination that could later interfere with image-based automated rotation estimation.

Second, because cells drifted throughout the course of an experiment, the script needed a frame of reference to translate an image patch, centered on the cell of interest, in order to decouple translation and rotation. A user manually entered an initial guess for the trajectory of the cell in a custom MATLAB GUI by clicking on the cell center periodically throughout the course of the video and interpolating to acquire intermediate approximate cell center positions. The script used these positions to seed an optimization that attempted to maximize the correlation between two image patches by finding the translation and rotation of the cell between the two patches.

Third, the script ran optimization routine to compute the frame-to-frame rotation rate (as well as small translations of the cell about its initial guess). The objective function is defined as follows. Given an (x, y) position (not necessarily integer pixels) of the center of the cell at one time (frame 1), interpolate between pixels to extract an image patch, P1, (27px × 27px) centered on that cell. Also, given a guess about the translation and rotation of the cell between this initial frame and the frame at a later time (frame 2), compute a set of translated-and-rotated pixel positions, and interpolate these positions on frame 2 to generate a second image patch, P2. To both patches, apply a blurred-edge circular mask (radius 7px, Gaussian blur radius 3px) to suppress the corners of the patches and include only pixels within the cell in subsequent steps. Compute the digital image correlation between the two masked patches (mP1 and mP2)

$$\rho = \frac{\sum_i \sum_j [mP1(i,j) - \overline{mP1}] [mP2(i,j) - \overline{mP2}]}{\sqrt{\left(\sum_i \sum_j [mP1(i,j) - \overline{mP1}]^2\right) \left(\sum_i \sum_j [mP2(i,j) - \overline{mP2}]^2\right)}}, \quad (1)$$

where (i,j) is a pixel within the patch, and $\overline{mP1}$ and $\overline{mP2}$ are the mean intensities of P1 and P2. Thus, the objective function optimizes over the arguments position and rotation, (Δx , Δy , θ), which determine the set of locations to interpolate from frame 2. MATLAB's fmincon tool

minimized the negative of this objective function. We set the tolerance in the change of the magnitude of the vector $(\Delta x, \Delta y, \theta)$ [px, px, degrees] to 10^{-6} . The initial guess for x and y originated from the manual clicking, and the initial guess for θ originated from the optimization of the previous two frames. Optimization constraints for translations were ± 5 px about the guess value supplied by manual clicking and for rotation was $\pm 49^\circ$ about the initial guess. Because `fmincon` is a gradient-based optimizer, it needed a cubic interpolation of the image intensity in order to make the objective function sufficiently smooth for proper convergence.

We have used a small applied voltage and a large electrode gap, leading to smaller applied field magnitudes and cell rotation speeds. Although this low field strength has the advantage of suppressing joule heating, cell rotation rates were so slow that cell rotation between adjacent frames was often sub-pixel, so angle displacements were compared over time increments of ~ 1 s, rather than 1 frame.

The final step of the rotation rate estimation is to break apart the ROT rate versus time over the whole video, as estimated from the angle displacement optimization at each frame, into the typical ROT rate within the 5 s window that each frequency was applied. We accomplished this synchronization by manually selecting a characteristic sharp peak at the beginning of a plot of accumulated angle versus time. We then took the median of all of the (potentially noisy) ROT rate measurements for each pair of frames in this each 5 s window as an outlier-resistant measure of the average ROT rate at that frequency. This automated ROT estimation correlated well with the manual ROT rate estimates from a custom MATLAB GUI in which a user clicked on a distinctive feature on a cell, tracking the feature as the cell rotated throughout the video (Figure 3).

E. Analytical model of ROT spectra

Fundamental equations for dielectrophoresis and electrorotation are described thoroughly in the literature.^{22–24,38} Briefly, complex permittivity depends on the substance permittivity, ϵ , conductivity, σ , and the angular frequency of the applied field, ω

$$\underline{\epsilon} = \epsilon + \frac{\sigma}{j\omega}. \quad (2)$$

FIG. 3. Estimated rotation rate of cells as measured by the automated image processing algorithm versus the rotation rate as measured by manually tracking features. Within each 5 s window that a particular field frequency was applied, approximately 37 frames were captured, yielding multiple measurements of the ROT rate. Each dot represents the median of such ROT rates in the window. The automated ROT rate estimation correlates strongly with the manual measurements, though slightly under predicting the manual ROT rate (least trimmed squares fit slope = 0.88 vs. ideal slope = 1).

A single spherical uncharged particle with homogeneous, isotropic conductivity and permittivity in a uniform sinusoidal external electric field perturbs the field equivalent to a dipole with moment phasor

$$\underline{\vec{p}}_0 = 4\pi \epsilon_m \underline{\vec{E}}_0 a^3 \frac{\epsilon_p - \epsilon_m}{\epsilon_p + 2\epsilon_m} = 4\pi \epsilon_m \underline{\vec{E}}_0 a^3 \underline{f}_{CM}, \quad (3)$$

where ϵ_m is the medium permittivity, $\underline{\vec{E}}_0$ is the external field phasor, a is the particle radius, ϵ_p is the particle effective complex permittivity, ϵ_m is the medium complex permittivity, and $\underline{f}_{CM}$ is the complex Clausius-Mossotti factor. For a sphere made of a thin membrane of zero conductivity and low permittivity compared to its contents (e.g., cytoplasm), the effective complex permittivity of that particle is

$$\epsilon_p = \frac{\epsilon_{cyto} C'' a}{\epsilon_{cyto} + C'' a}, \quad (4)$$

where C'' is the membrane capacitance per unit area and ϵ_{cyto} is the complex permittivity of the contents. This dipole interacts with a non-uniform external field to give rise to the familiar time-averaged dielectrophoresis force

$$\langle \vec{F}_{DEP} \rangle = \pi \epsilon_m a^3 \text{Re}(\underline{f}_{CM}) \nabla (\underline{\vec{E}}_0 \cdot \underline{\vec{E}}_0), \quad (5)$$

which is proportional to the real part of the Clausius-Mossotti factor. The dipole may also interact with a rotating external field to yield a time-averaged torque

$$\langle \vec{T}_{ROT} \rangle = -4\pi \epsilon_m a^3 \text{Im}(\underline{f}_{CM}) [\text{Re}(\underline{\vec{E}}_0) \times \text{Im}(\underline{\vec{E}}_0)], \quad (6)$$

which is proportional to the negative of the imaginary part of the Clausius-Mossotti factor. In the Stokes flow limit, the viscous torque, proportional to cell rotation rate, instantly balances the ROT torque. Thus, the imaginary part of the Clausius-Mossotti factor (which varies with applied field frequency) is proportional to the observed cell rotation rate at that applied frequency

$$\text{Im}(\underline{f}_{CM}(\omega)) \propto \Omega(\omega). \quad (7)$$

With a detailed model of the applied field strength and the viscous hydrodynamics, we could directly solve $\text{Im}(\underline{f}_{CM})$, but such modeling challenges may be avoided by including a scale factor accounting for field strength and the viscous hydrodynamics in the curve fit.

F. Robust parameter fits to ROT measured ROT spectra

In the inertia-free limit of Stokes flow, ROT rates are proportional to the imaginary part of the Clausius-Mossotti factor. Rather than attempting to model the complicated friction between the cell and the wall and the precise magnitude of the electric field at each point in the ROT chamber, we fit ROT spectra to $\text{Im}(\underline{f}_{CM})$ up to a scale factor, which accounted for the field strength and friction. In addition to the scale factor, the parameters also used to make the fit are the specific membrane capacitance, the cytoplasmic conductivity, and the cytoplasmic permittivity.

Despite carefully designing an image processing algorithm and using the median within each frequency window to suppress outliers, the measured ROT spectra occasionally contain a few outliers. These outliers can strongly influence curve fits (Figure 4), so the nonlinear least trimmed squares were selected as an outlier-resistant curve fitting scheme.³⁹ To conduct least trimmed squares, an initial guess of parameters generates a model ROT rate for each frequency,

FIG. 4. A least trimmed squares fit and a traditional nonlinear least squares fit showing the influence of outliers on the two curve fits on a ROT spectrum. The direction of the y-axis is reversed to make this plot of rotation rate consistent with the imaginary part of the Clausius-Mossotti factor, which has the opposite sign.

$\Omega_{model}(\omega_i, params)$. The squared deviation of the model from the data is then computed for each point in the spectrum

$$g_i = (\Omega_{meas}(\omega_i) - \Omega_{model}(\omega_i, params))^2. \quad (8)$$

A priori, the user had decided some number of the points to trim from the fit (3 for our work). Points that deviate the most are then trimmed from the dataset, and a new model is computed via standard nonlinear least squares. A new set of g_i 's is computed (now including the trimmed points). This process iterates until the same set of points are trimmed between iterations.

We used MATLAB's `fmincon` to do the intermediate nonlinear least squares fit, but rather than fitting σ_{cyto} , ϵ_{cyto} , C''_{mem} , and, directly, the logs of these quantities were fit to the curve. This ensured that (1) the physical quantities always remained positive, (2) the quantities were all of a similar order of magnitude, and (3) the sensitivity of fit parameters to the data varies on a log scale as the data points are spaced in log frequency. The scale parameter was not fit on a log scale. The guesses used to initialize all parameters were: scale = -0.5269 rad/s, $\sigma_{cyto} = 0.5299$ S/m, $\epsilon_{cyto} = 93.1$, and $C''_{mem} = 19.9$ mF/m², which were median parameters computed from an initial optimization of the data. Constraints on the parameters were scale $\in [-10, 10]$ rad/s, $\sigma_{cyto} \in [0.1, 100]$ S/m, $\epsilon_{cyto} \in [10, 1000]$, and $C''_{mem} \in [0.15, 1500]$ mF/m², well beyond the physical range of these parameters that one would expect from the literature. We excluded cells where the optimization of at least one property converged to a constraint, omitting them from further analysis.

III. RESULTS AND DISCUSSION

Our device applied rotating electric fields of various frequencies to cancer cells and white blood cells, and an image processing script estimated subsequent rotation rates of cells automatically from the captured video. Because of the cells' minimal inertia, viscous forces dominate and their rotation rate is proportional to the electrorotation torque. The electrorotation torque is proportional to the imaginary part of the Clausius-Mossotti factor, which depends on the permittivity and conductivities of the cell cytoplasm and the specific capacitance of the cell membrane. With an additional measurement of the radius of the cell measured from the bright field images, we inferred these cell electrical properties (along with a proportionality factor accounting for electric field magnitude and viscous torques) from curve fits of the cells' measured ROT spectra. ROT measurements are, by nature, single cell, enabling not only measurements of

average cell properties, but also quantification of the distribution of cell properties. Figure 5 highlights the spread of fit ROT spectra, allowing for a comparison of cells within each population. Most spectra consist of two relaxations that appear as a peak and a trough: (1) a low frequency ($\sim 10^5$ Hz) peak dominated by the cell radius, specific membrane capacitance, and suspending medium conductivity, and (2) high-frequency ($\sim 10^6$ – 10^8 Hz) trough associated with DEP transitioning from being dominated by the cell-medium conductivity mismatch to the cell-medium permittivity mismatch.⁴⁰

The effect of acquired resistance to gemcitabine on BxPC3 pancreatic cancer cell sub-clones appears in Figure 6. Most notably for the design of future DEP devices, the calculated crossover frequency significantly decreased for both gemcitabine-resistant sub-clones as compared to gemcitabine-naïve parental BxPC3s ($p < 0.05$, Wilcoxon rank sum test), effectively widening the frequency window at which a majority of cancer cells experience pDEP and a majority of white blood cells (WBCs) experience nDEP. (Also see Figure 9). Because the time constant associated with this relaxation is approximately proportional to the product of specific membrane capacitance and cell radius, the observed decrease in crossover frequency is explainable due to the observed increase in median cell radius and in median specific membrane capacitance of the gemcitabine-resistant sub-clones (Table I). Wang *et al.*,⁴¹ Shim *et al.*,⁸ and Gascoyne *et al.*⁴² attribute higher specific membrane capacitances to larger effective membrane areas due to ruffles and folds in the membrane, changing the apparent effective specific capacitance. Alternatively, Gagnon *et al.*⁴³

FIG. 5. Fit electrorotation spectra. Each faint curve depicts the imaginary part of the Clausius-Mossotti factor fit to an individual cell of a particular type. Bold curves represent a theoretical cell with cytoplasmic conductivity, cytoplasmic permittivity, membrane specific capacitance, and radius equal to the median fit values for the cells of that type.

FIG. 6. (a)–(f) Scatterplots of the electrical properties (fit from electrorotation spectra) for BxPC3s and BxGRs. Each point corresponds to the electrical properties obtained from an individual cell’s electrorotation spectrum. (g) Representative computed dielectrophoresis (DEP) spectra of BxPC3 pancreatic cancer cells and two gemcitabine-resistant BxPC3 sub-clones (a moderately resistant “BxGR 80c” and a strongly resistant “BxGR 360c”) obtained by growing generations of BxPC3s in increasing concentrations of gemcitabine. Curves generated by taking the median of the cell electrical properties obtained from the cell type’s electrorotation spectra fits. Note that the spectra for the two gemcitabine-resistant sub-clones nearly overlap. The analogously obtained white blood cell fit properties (a)–(f) and computed DEP spectrum (g) are included for reference.

observed that crosslinking agents could change the effective permittivity of cell membranes. Because both cell membrane protein composition as well as the gross physical phenotype of drug-resistant pancreatic cancer cells become dysregulated,¹³ both ruffling and permittivity changes may contribute to the observed membrane capacitance change.

If the trend of a larger specific membrane capacitance for gemcitabine-resistant cancer cells also exists *in vivo*, then the separation potential would exist even noting that (for prostate cancer) CTCs tend to be smaller than their cell line counterparts.⁴⁴ These gemcitabine-resistant clones developed one of many potential resistance mechanisms. It is possible that clones that have acquired resistance via different mechanisms may exhibit gross physical changes that have a less favorable impact on their crossover frequency in terms of the remaining that are separable from blood cells. Studying such clones will be the subject of future work.

Figure 7 shows the effect of serum starvation on BxPC3 and PANC1 cells. Serum-starving PANC1 cells significantly increased their crossover frequency, though the crossover frequency still remains significantly lower than that of WBCs (see Table I and Figure 9, $p < 0.05$,

FIG. 7. (a)–(f) Scatterplots of the electrical properties (fit from electrorotation spectra) for each cell type and serum condition. Each point corresponds to the electrical properties obtained from an individual cell's electrorotation spectrum. (g) Representative computed dielectrophoresis (DEP) spectra of serum-starved and non-serum-starved pancreatic cancer cell lines (both BxPC3 and PANC1). Note that the spectrum for BxPC3s overlaps with the spectrum for serum-starved PANC1s. Curves generated by taking the median of the cell electrical properties obtained from the cells' electrorotation spectra fits. The analogously obtained white blood cell fit properties (a)–(f) and computed DEP spectrum (g) are included for reference.

Wilcoxon rank sum tests). No significant change in crossover frequency was observed for serum-starved BxPC3 cells as compared to their non-starved controls, despite the duration of serum starvation (48 h) being longer than protocols used to cause vesicle blebbing from the cell membrane (overnight)⁴⁵ or longer than the maximum lifetime of cancer cells in circulation (<24 h).⁴⁶ The lack of an observed change in crossover frequency is likely due to our observation that cell radii tended to increase with serum starvation while specific membrane capacitance tended to decrease.

We implemented an EMT protocol modeled on work from the literature.⁴⁷ Our EMT-induced cells had spindle-like morphology and a pre-liftoff surface area that was larger than the untreated PANC1 cell surface area (Figure 8(h-i)), as well as a downregulation of epithelial proteins with an upregulation of hallmark mesenchymal markers (confirmed by immunofluorescence, data not shown). Furthermore, previous investigators^{13,28,48} have observed a strong connection between gemcitabine resistance in pancreatic cancer cells and the acquisition of mesenchymal characteristics, including an increase in pre-liftoff surface area. Thus, we had

TABLE I. Medians, (25–75 percentiles), and {95% bootstrapped confidence intervals} of electrical properties of white blood cells and cancer cell lines. Cytoplasmic conductivity, cytoplasmic relative permittivity, and membrane specific capacitance were computed by nonlinear curve fits to electrorotation spectra. Cell radii were measured by manually marking bright field images of cells. Crossover frequencies were computed for each cell according to its fit properties. Medium of conductivity was measured to be 70 mS/m and relative permittivity was calculated based on Ref. 31 to be 74.12. BxGR80c and BxGR360c are moderately and strongly gemcitabine resistant sub-clones (respectively) of BxPC3.

	Membrane specific capacitance [mF/m ²]	Cell radius [μm]	Cytoplasmic conductivity [mS/m]	Cytoplasmic relative permittivity []	Calculated crossover frequency [kHz]	Significant ^a difference in crossover freq. from WBC?	Significant ^a difference in crossover freq. from corresponding untreated cell?
White blood cells (N _{cells} = 49)	9.8 (8.1–11.9) {9.0–11.0}	5.6 (5.4–5.9) {5.5–5.8}	721 (530–977) {610–886}	111 (66–135) {79–122}	271 (224–334) {250–294}	Base type	No comparison made
U937 (Lymphoma cells) (N _{cells} = 57)	14.0 (11.4–17.3) {13.0–15.4}	7.5 (6.9–8.3) {7.2–8.0}	750 (606–903) {663–839}	106 (85–131) {95–118}	147 (113–172) {132–163}	Yes	No comparison made
PANC1 (Pancreatic cancer cells) (N _{cells} = 97)	20.2 (14.4–28.6) {18.3–23.3}	12.1 (10.9–13.9) {11.7–12.6}	476 (328–607) {424–530}	90 (69–131) {77–101}	64 (44–80) {56–67}	Yes	Base type
Serum-starved PANC1s (N _{cells} = 53)	15.4 (12.2–23.9) {13.2–18.1}	12.6 (10.7–13.6) {11.7–13.1}	462 (355–622) {401–530}	134 (81–216) {96–163}	81 (55–106) {63–92}	Yes	Yes
EMT-induced PANC1s (N _{cells} = 29)	20.1 (15.9–27.5) {16.8–24.9}	12.7 (11.5–15.8) {11.8–15.7}	536 (352–690) {442–647}	91 (66–107) {72–103}	54 (40–73) {44–66}	Yes	No
BxPC3 (Pancreatic cancer cells) (N _{cells} = 101)	22.5 (19.6–28.7) {21.3–23.9}	8.1 (7.4–9.1) {7.9–8.4}	453 (320–571) {391–504}	91 (72–111) {84–98}	80 (61–98) {72–83}	Yes	Base type
Serum-Starved BxPC3 Cells (N _{cells} = 39)	21.7 (18.8–26.1) {19.5–23.4}	8.5 (7.7–9.4) {8.0–9.2}	467 (417–568) {446–521}	88 (74–108) {76–94}	82 (69–98) {75–88}	Yes	No
BxGR80c, strongly gemcitabine resistant subclone (N _{cells} = 55)	26.4 (22.6–29.6) {25.0–28.6}	9.9 (9.0–10.8) {9.5–10.2}	503 (395–608) {459–572}	85 (70–102) {81–94}	56 (50–70) {52–64}	Yes	Yes
BxGR360c, strongly gemcitabine resistant subclone (N _{cells} = 54)	25.9 (22.6–31.0) {24.5–28.5}	9.6 (8.5–10.1) {9.0–9.8}	575 (470–673) {525–611}	92 (72–105) {81–99}	59 (53–74) {55–65}	Yes	Yes

^aMedian calculated crossover frequency of the cell type/treatment significantly differs ($p < 0.05$, Wilcoxon rank sum test) from that of the white blood cells (left column) or untreated cells of the same type (right column).

expected to find a strong difference between the EMT-induced PANC1 cells and their untreated controls, due to this morphology change. According to Wang *et al.*,⁴¹ Shim *et al.*,⁸ and Gascoyne *et al.*,⁴² this increase in surface area would putatively change the ruffles and folds in the cell membrane, increasing their effective surface area and specific membrane capacitance. Furthermore, Salmanzadeh *et al.*⁴⁹ and Mulhall *et al.*⁵⁰ observed that aggressiveness of cancer cell phenotype correlates with an increase in specific membrane capacitance for mouse ovarian surface epithelial and oral cancer cells, respectively. We, however, observed no significant change in the electrical properties of EMT-induced PANC1 cells. Figures 8(a)–8(g) show the effect of EMT induction on the DEP spectra and electrical properties of PANC1 cells. No

FIG. 8. (a)–(f) Scatterplots of the electrical properties (fit from electrorotation spectra) for untreated and EMT-induced PANC1s. Each point corresponds to the electrical properties obtained from an individual cell’s electrorotation spectrum. (g) Representative DEP spectra of untreated PANC1 pancreatic cancer cells and PANC1s that have been treated with to induce the epithelial-to-mesenchymal transition (EMT). Curves generated by taking the median of the cell electrical properties obtained from the cells’ electrorotation spectra fits. The analogously obtained white blood cell fit properties (a)–(f) and computed DEP spectrum (g) are included for reference. (h)–(i) Phase contrast images of the PANC1 cells (h) and the EMT-induced PANC1 cells (i), adhered to culture flask prior to liftoff.

significant change was observed in the crossover frequency of the EMT-induced cells compared to their non-induced counterparts (Table I). Regardless of EMT treatment, PANC1 cells’ crossover frequency remained significantly lower than that of WBCs (Table I, Figure 9).

Cytoplasmic permittivity could also be inferred from the ROT spectra. Conductivities for pancreatic cancer cells tended to be lower than U937 Leukemia Cells or white blood cells. These differences could have arisen from many possible factors including different cell lineages, different growth conditions, dysregulation of ion concentration due to altered cell metabolism or cell signaling, differences in ion permeability of interior cell structures, or differences in the concentration of non-ionic species. Mulhall *et al.* distinguishes between non-cancerous and cancerous oral cells based on their cytoplasmic conductivity,⁵⁰ noting that decreased cytoplasmic conductivity is associated with progression.

ROT measurements enable simultaneous fits of multiple cell properties, compared to crossover frequency measurements, which allow for fitting of only one parameter (typically specific

FIG. 9. Fraction of cells predicted to experience positive DEP as a function of applied field frequency, displayed by computing the CDF of experimentally measured cells whose ROT spectra predict a positive DEP response. Predicted DEP crossover frequencies are computed cell by cell from fit ROT spectra. In a window of approximately 100 kHz–200 kHz, nearly all pancreatic cancer cells (regardless of treatment) are predicted to experience positive DEP while minimal white blood cells also experience positive DEP.

membrane capacitance). Because of this increased information, we were able to make estimates of the cells' cytoplasmic relative permittivity. White blood cell median permittivity was measured to be 111 (95% bootstrapped confidence interval 79–122), and typical median pancreatic cancer cell relative permittivity was measured to be approximately 96 (see Table I). These values are higher than those from ROT experiments of T lymphocytes and MDA321 breast cancer cells (64 and 52) reported by Becker *et al.*¹⁶ and used by many subsequent studies.^{14,17,18,51} The dielectric increment of glycine in water is positive,^{23,52} whereas the dielectric increment of salts is negative,^{23,53} so their competing effect on the permittivity of cytoplasm is unclear. The concentration of either type of solute could be different in cells with a highly dysregulated growth and metabolism.

Parasitic impedance in the device and high-frequency attenuation of the prescribed signal in the generator could have led to a weakening of the high-frequency ROT response of the cells. A smaller high-frequency ROT peak would cause us to under-predict the strength of the transition from DEP dominated by conductivity mismatch (10^6 Hz– 10^7 Hz) to DEP dominated by permittivity mismatch ($>10^8$ Hz),⁴⁰ and thus over-predict cytosolic permittivity. Because the fit relative permittivity is higher than that of the suspending medium (74.2), these data predict that no upper crossover frequency exists for these cells. Unfortunately, the upper crossover frequency predicted by the low permittivity values in the literature was higher than our experimental apparatus can generate, so we could not validate this prediction. Nonetheless, permittivity has a negligible impact on the low-frequency DEP behavior, so our conclusions about (lower) crossover frequency of cancer cells and blood cells remain unaffected by this experimental uncertainty.

IV. CONCLUSION

By using rotating electric fields to induce frequency-dependent-torques on polarizable particles (electrorotation), we have inferred the electrical properties (cytoplasm permittivity and conductivity as well as cell membrane specific capacitance) of pancreatic cancer cells and blood cells by their observed rotation rates at each field frequency. Electrorotation measurements are by nature single-cell, and this study has revealed the distribution of electrical properties within cancer cell line populations. For all pancreatic cancer cells measured and under all measured treatments of the cells (acquired resistance to gemcitabine, serum starvation, and induced epithelial-to-mesenchymal transition), a significant window exists within which at least a majority of cancer cells exhibit positive DEP while a majority of blood cells exhibit negative DEP. The robustness of the distinguishing electrical properties to the applied treatments is promising for DEP-enhanced cell capture devices, as proposed in the other work.^{17,18,20} Furthermore, these

measurements revealed a higher cytoplasmic permittivity than has been previously reported in the literature, which predicts that no upper crossover frequency exists and could be the study of future investigation. Future work could involve studying ROT of cancer cells at higher frequencies to validate this measurement and DEP at higher frequencies to exploit permittivity mismatches to separate cells. Additional future work might involve trying single cell expression levels of various markers (e.g., for EMT) and the corresponding cells' ROT spectra by taking an immunofluorescence image prior to capturing ROT videos, which would strengthen the connection between single cell electrical properties and expression levels of markers of interest.

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