Label-Free Automated Cell Tracking: Analysis of the Role of E-cadherin Expression in Collective Electrotaxis

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Abstract—Collective cell migration plays an important role in wound healing, organogenesis, and the progression of metastatic disease. Analysis of collective migration typically involves laborious and time-consuming manual tracking of individual cells within cell clusters over several dozen or hundreds of frames. Herein, we develop a label-free, automated algorithm to identify and track individual epithelial cells within a free-moving cluster. We use this algorithm to analyze the effects of partial E-cadherin knockdown on collective migration of MCF-10A breast epithelial cells directed by an electric field. Our data show that E-cadherin knockdown in free-moving cell clusters diminishes electro-tactic potential, with empty vector MCF-10A cells showing 16% higher directedness than cells with E-cadherin knock-down. Decreased electrotaxis is also observed in isolated cells at intermediate electric fields, suggesting an adhesion-independent role of E-cadherin in regulating electrotaxis. In additional support of an adhesion-independent role of E-cadherin, isolated cells with reduced E-cadherin expression reoriented within an applied electric field 60% more quickly than control. These results have implications for the role of E-cadherin expression in electrotaxis and demonstrate proof-of-concept of an automated algorithm that is broadly applicable to the analysis of collective migration in a wide range of physiological and pathophysiological contexts.

Keywords—Cell–cell interactions, Electrotaxis, Guidance cues, Image analysis.

INTRODUCTION

Collective migration is required in many physiological processes including tissue development and repair.^{3,14} Meanwhile, collective migration is an important factor in metastasis, wherein cancer cells

move in groups or streams, or benign cells co-migrate with invasive cells within a tumor microenvironment.^{8,10,15} Cell migration, both collective or of isolated cells, is directed by gradients in a variety of applied stimuli including chemical factors, mechanical properties, and electric fields.^{28,35,50} Often, migratory patterns arise when cells migrate collectively that would not be predicted from the movement of individual cells. For example, MDCK cells have been shown to undergo collective electrotaxis despite the fact that individual cells were not observed to migrate within an electric field in isolation.²² To understand the quantitative features of collective migration, it is essential to acquire and analyze positional data of a large number of migrating cells both in isolation and within cell clusters.

The standard approach of cell tracking involves manually identifying the center of a migrating cell or its nucleus in every frame of a time-lapse series of microscopic images. While this approach yields a direct measure of cell migration trajectories, there are a number of drawbacks. A manually recorded path of a cell will likely vary depending on the person tracking it. Even the same cell tracked by the same person is not guaranteed to be reproducible. Moreover, manual tracking is laborious and time-consuming.

In contrast, automated tracking offers the potential for reproducible, rapid, and efficient acquisition of migration trajectories. Many algorithms for cell and particle tracking have been developed, but most require additional labeling steps to function, such as magnetic resonance² or fluorescence.⁴⁹ For example, open access platforms such as ImageJ and Fiji provide tracking algorithms such as TrackMate³⁹ and the MOSAIC Suite,³⁸ but both are designed optimally for fluorescently-labeled cells or cell parts. Fluorescent labeling aids automated tracking by localizing fluo-

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rescent signal to the nucleus³³ or another organelle thereby increasing the contrast between regions of interest and the background. Nuclear tracking can be implemented with dyes that associate with DNA such as Hoechst²¹ or a histone 2B-GFP fusion protein such as H2BGFP.³⁶

Fluorescent labeling, however, is not without drawbacks. It is widely known that exposure to visible and ultraviolet light is toxic to mammalian cells in culture.⁶ Stable fluorescent labeling of proteins is commonly done *via* viral transduction, which requires microbiological techniques and equipment. Commonly used fluorescent dyes, while simple in application, lose fluorescence with age as cells divide and distribute dye between daughter cells. Site-specific fluorescent antibodies are expensive and often require fixation and permeabilization of cells in order to visualize internal cell components.

An ideal solution to track cell migration involves the development of automated algorithms capable of processing phase-contrast images of label-free cells. Such an algorithm would significantly simplify experimental protocols while providing robust data processing. Tracking label-free cells within clusters is not straightforward because of the low level of contrast at cell boundaries. There are methods which increase the contrast between cells. For example, third-harmonic generation (THG) offers the ability to analyze fluids close to lipid membranes¹² and has been used for tracking lineage of cells within the zebrafish blastocyst, where fluorescent staining would be too difficult.³¹ Ptychography, which enhances contrast by comparing diffraction patterns to brightfield images, has recently garnered attention as a label-free imaging technique.²⁴ Multi-photon techniques such as THG and multiple camera techniques are often unavailable for the typical biological laboratory, whereas phase-contrast microscopy is ubiquitous in tissue culture facilities.

There has been significant progress in automating the analysis of phase-contrast microscopy images. The automation of identifying even isolated, single cells has proved challenging, but can be accomplished using trained background subtraction and edge detection.⁹ The issue of separating adjacent cells remains an issue and is not easily overcome without combining fluorescent imaging of intercellular components such as the nucleus.⁴⁵ Even in a crowded environment, morphological properties of cells can be detected using Fourier transform based feature detection.¹ While this is useful, especially in high-throughput drug screening, the spatiotemporal location of cells analyzed in this method is not produced. Using a morphological watershed, cell boundaries can be detected but often require additional, computationally intense post-processing steps.⁴⁶ Despite these advances, the spatial

resolution for segmenting cells in a clustered or crowded environment is still poor; in fact, relying solely on phase-contrast images typically provides only enough resolution to differentiate between regions of one cell type versus another.¹⁸

Here, we develop a label-free tracking algorithm capable of identifying individual cells within a migrating cell cluster. A trademark of the method described herein is that images are cropped into multiple, overlapping images in such a way to increase the robustness of image processing techniques. Individual cells are tracked sequentially through frames so that the previous location can be used to infer the location of a region of interest.

We apply this algorithm to study the electrotaxis of clustered epithelial cells in a high throughput manner. We and others have previously shown that clustered cells exhibit better electrotactic response than isolated counterparts.^{20,22} We sought to investigate the role of the expression of E-cadherin, a cell surface receptor that mediates cell–cell adhesion,^{40,44} in the enhanced electrotaxis of clustered cells. E-cadherin expression is often downregulated in cancer progression^{30,41} and epithelial-derived cancer cells typically display robust electrotaxis in tissue culture.^{22,48,50} The association between metastatic potential and electrotaxis may be a result of the endogenous electric field which arises at the interface of cancerous and non-cancerous tissue.^{11,25} Indeed, endogenous electric fields have been measured in a clinical setting and were demonstrated to provide a diagnostic modality for detecting breast cancer.¹¹ Moreover, inhibition of E-cadherin with DECMA-1 or by Ca^{2+} depletion eliminated electrotaxis in clusters of MDCK cells.²² To increase the throughput of our analysis, we develop a label-free tracking algorithm capable of identifying individual cells within a migrating cell cluster.

The algorithm is used to track and analyze electrotaxis of breast epithelial MCF-10A cells, which were chosen because electrotaxis of MCF-10A cells is not dependent on the presence of cell–cell junctions. We extensively validate this algorithm and find it to perform comparably to interfaces for manual tracking. We find that a 40% knockdown in E-cadherin expression decreases the alignment of migration within an electric field by approximately 20%. This decrease in migration alignment is observed for both clustered cells and isolated cells, suggesting an adhesion-independent role of E-cadherin in sensing and responding to electric fields. Additionally, reduction of E-cadherin expression alters the kinetics of alignment with electric fields by increasing the sensitivity to electric field magnitude of clustered cells and increasing the speed of realignment of isolated cells. These findings help elucidate the interplay between loss of E-cadherin in

cancer progression and the ability to sense and respond to the electric microenvironment associated within a developing tumor.

MATERIALS AND METHODS

Cell Culture

MCF-10A cells transduced with retrovirus encoding either an empty vector control (10A-pLKO) or a vector encoding E-cadherin shRNA (10A-shEcad) were provided by Senthil Muthuswamy at Harvard Medical School and used as previously described.²⁷ Cells were cultured in growth medium composed of Dulbecco's modified Eagle's medium/Ham's F-12 containing HEPES and L-glutamine (DMEM/F12, Invitrogen) supplemented with 5% horse serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen), 10 $\mu\text{g/mL}$ insulin (Sigma), 0.5 $\mu\text{g/mL}$ hydrocortisone (Sigma), 20 ng/mL EGF (Peprotech) and 0.1 $\mu\text{g/mL}$ cholera toxin (Sigma) and were maintained under humidified conditions at 37 °C and 5% CO₂. Cells were passaged as described previously¹⁹ and were discarded after passage 35.

Device Fabrication

The electrotactic chamber was assembled similarly to that described by Song⁴² with modifications described in our previous work.²⁰ Briefly, polystyrene dishes (60 mm) were taken and marked with lines 12 mm apart. Number 1 glass coverslips (22 $\times$ 22 mm²) were cut in half and then attached to either side of the lines using DC4 silicon grease (Dow Corning), leaving a 12 mm gap between them to produce a 12 mm $\times$ 22 mm cell seeding region on the dish. Barriers were constructed orthogonally from the edge of the glass coverslips using 3140 silicon adhesive (Dow Corning) in order to produce two media reservoirs on either side of the 12 mm $\times$ 22 mm gap. Dishes were then left to dry for at least 12 h while being sterilized under ultraviolet light and were then stored for up to 4 weeks.

Polydimethylsiloxane (PDMS) was synthesized by mixing prepolymer to cross-linker in a 10:1 ratio. The solution was degassed in a vacuum chamber until expulsion of bubbles ceased. The mixture was then cured at 80 °C for 1 h in an unmodified plastic dish (100 mm diameter). Wells were cut from PDMS blocks so as to fit into the region between the coverslips. The wells were sterilized under UV light.

Device Preparation

Dishes were then coated with a 10 $\mu\text{g/mL}$ solution of fibronectin (Invitrogen) in PBS (Invitrogen) for 1 h

prior to cell seeding. The solution of epithelial cells (500 μL) was added to the PDMS well at varying concentrations ($\sim 10^3$ – 10^5 cells/mL), and cells were left to adhere for at least 12 h in incubation. After rinsing with growth media to removed non-adherent cells, a coverslip roof was attached to the chamber *via* DC4 silicon grease in order to define a chamber with dimensions 12 mm $\times$ 22 mm $\times$ 0.15 mm. After closing the chamber, the medium was replenished once more before imaging.

Image Acquisition

Imaging was performed on an AxioVert 200 M inverted microscope (Zeiss). Devices were maintained at 37 °C and 5% CO₂ for 6 h. Glass tubes (6" $\times$ 7 mm ID) were bent under flame to fit beneath the condenser of the microscope. Current was delivered to the chamber by agar bridges made by filling the glass tubes with a 5% (w/v) solution of agarose (EMD) dissolved in heated serum-free media and left to cool and solidify at 37 °C. The use of serum-free media in the agar bridge stabilized the pH of the media throughout the 6 h experimental window. Current was generated by a WaveNowXV potentiostat (Pine) connected to disposable aluminum electrodes resting in reservoirs of 1 M KCl solution. The height of the KCl reservoirs were kept approximately equal to that of the medium in the device to prevent syphoning of fluid. After 6 h, no syphoning of KCl into the electrotactic chamber was observed. Images were acquired by phase-contrast microscopy every 5 min.

Image Analysis

Time-lapse videos were processed using a custom tracking interface written in MATLAB (MathWorks, Natick, MA) in order to extract the two-dimensional position of cells with respect to time. For cell cluster experiments, a custom script was written that accurately identified and tracked cells with $\sim 80\%$ efficiency, defaulting to manual tracking when necessary (Fig. 1). The MATLAB script and GUI are provided in the Supplemental Information. Isolated cells were tracked with the manual tracking interface reported previously.²⁰ Cells which divided, died, detached from the substrate, or were otherwise compromised during the middle of the video were discarded from analysis. In the case of isolated cells, cells which were observed to interact with other cells were discarded from analysis. Cells which departed the field of view were tracked until that time, and positional data up until the time of departure were compiled with the remainder of the data.

Quantification of Cell Migration

Cell trajectories were analyzed further using MATLAB to assess several properties of migration which have been reported previously.

Directedness (D) was calculated using Eq. (1),

$$D = \cos(\theta) \quad (1)$$

where θ is the angle formed between the cell migration vector and the direction of the electric field with the cathode as the origin and anode as the terminal. By this definition, a directedness value of 1 or -1 would be indicative of a net directional bias toward the anode or the cathode, respectively.

Persistence was calculated from Eq. (2),

$$P = x/l \quad (2)$$

where l is the path length of a cell and x is the net displacement over the same duration. For a cell migrating in a perfectly straight line, x and l will be equal, so P will be unity.

We have previously introduced the characteristic orientation time as a metric of electrotactic reorientation speed. Cells are filtered based on initial trajectory (which is stochastically governed) in order to determine the subset of cells initially moving in the opposite of the expected steady-state direction. The evolution of directedness of these cells, $D(t)$, was then fit to the following exponential recovery model using MATLAB's `nlfit`:

$$D(t) = (D_{ss} - D_{min})(1 - e^{-\lambda t}) + D_{min} \quad (3)$$

where D_{ss} is the steady-state value of directedness at a particular set of conditions, D_{min} is the nadir of the curve, λ is orientation coefficient (min^{-1}), and t is the time (min). The characteristic time needed to reach half maximal orientation (τ) is related to the orientation coefficient by the following equation:

$$\tau = \log(2)/\lambda \quad (4)$$

Statistical analysis was done using the Statistics Toolbox in MATLAB.

RESULTS

Development of Algorithm and User Interface for Tracking of Clustered Cells

Since cell clusters have a high number of cells per unit area and the number of cell clusters analyzed must be sufficiently large for statistical robustness, the number of individual cells that must be tracked quickly grows into the thousands. In order to increase the throughput of analysis for these experiments, we developed an automated approach to quantify migra-

tion trajectories of clustered cells. To monitor the automated analysis so that a user could intervene manually when necessary, a custom user interface was written in MATLAB (MathWorks, Natick, MA) (Supplement Fig. 1). The user interface begins by requesting the user to identify the initial positions of every cell in the initial frame of the time-lapse series. Unlike most tracking algorithms, which analyze one frame at a time then perform frame-to-frame pairing to compile complete cell tracks, this algorithm focuses on one cell at a time. Its movement trajectory is determined longitudinally over time to the end of the video, and then the algorithm returns to the initial frame to process the next cell. In doing so, image analysis techniques, such as black-white thresholds, are not influenced by the pixel intensity outside the immediate proximity of the cell of interest. Additionally, portions of each sequential frame are analyzed multiple times so, rather than attempting to acquire all relevant data on a single pass of the algorithm, data can be acquired and categorized on a per cell basis with a high degree of robustness.

A step-by-step walk through of how the algorithm segments and identifies the position of a cell is provided in Fig. 1. First, the portion of the frame surrounding the initial coordinates of the cell is cropped for analysis (Fig. 1a). By focusing only on the cell of interest and the immediately surrounding area, the robustness of image processing techniques is enhanced and the computational time required for processing is decreased. The contrast of the cropped image is enhanced by using a log-scale conversion (Fig. 1b). This helps to highlight cell junctions in order to distinguish between adjacent cells. Intracellular structures are highlighted by estimating derivative of pixel intensity of the contrast enhanced image in 2 dimensions and is stored separately (Fig. 1c). Then, a binary mask is constructed from the contrast enhanced image based on intensity (Fig. 1d). The pixel intensity threshold for the binary mask is determined automatically for the contrast enhanced image. Since the image is contrast enhanced, the process of cropping around the area of interest is critical to prevent far off regions from influencing the threshold determination. This binary mask is combined with the contrast enhanced image to remove regions initially determined to be cell-cell boundaries (Fig. 1e). After merging with the mask, regions of interest are further separated by morphological erosion using a one pixel disk structural element, which serves to remove roughness at the region borders (Fig. 1f). This image is then merged with the highlight of intracellular structures, the result of which is increased pixel intensity towards the interior of the cell (Fig. 1g). A second thresholding is performed on this compound image in order to remove more space

FIGURE 1. Algorithm workflow for image processing. (a) Original phase microscopy image of cell cluster focused on a relatively small area. The center of the focused area is determined by the initial point selected by the user or by the centroid of the cell from the previous frame. (b) Logarithmic contrast-enhanced image. This step helps increase intensity within the cell interior relative to the cell boundaries. (c) Feature edge detection of contrast enhanced image *via* estimation of the intensity derivative. Edges are predominately centered around cell boundaries, nuclei, and vacuoles. (d) Black-white mask generated from contrast enhanced image. (e) Combination of mask with original contrast enhanced image to remove most of cell boundaries from the image. (f) Opening (dilation and subsequent erosion) of masked image. Opening of the image removes a large fraction of cell-cell junctions. (g) Combination of edge detection with opened image. This step plays an important role by increasing the intensity of the image towards the cell center. (h) Opening of combined image. This further removes areas which would have been close to the cell boundaries. (i) Size filtering to reduce detected fragments and background noise. (j) Identified cell center based on distance limitation is labeled with a white circle and white arrowhead. Scale bars on [shown on (a) and (j)] are 50 microns.

between cells, also using an automatically determined threshold (Fig. 1h). Since the highlighting of intracellular structures tends to create ring-like regions of intensity, the interior of such regions is filled to generate solid regions of interest. To finish, the image is morphologically closed with a two pixel disk structural element followed by a size limited filter (at $10\times$ magnification, 50 pixels was used as the upper limit of noise) to remove any remaining background or cell fragments from being analyzed (Fig. 1i). The coordinates of the cell center are determined as the centroid of the detected region of interest (Fig. 1j). When multiple regions of interest (ROI) are detected, which tends to occur when the nuclear area is large compared to cell area, the centroid closest to the location of the cell centroid in the previous frame is chosen. This approach for resolving multiple ROIs assumes the cell has moved a minimum distance and is accurate provided the time interval between frames is small relative to the time scale of cell movement.

The segmentation process outlined in Fig. 1 is repeated sequentially through each frame acquired by time-lapse microscopy. At each successive time point, the sub-field around the cell of interest is cropped for analysis based on the coordinates of the recorded cell centroid from the previous frame. Once the new position is determined, several checks are performed on the displacement and size of the segmented cell to confirm that the cell identified in the new frame corresponds to the same cell in the previous frame. Specifically, recorded cell speed may not exceed $1.3\ \mu\text{m}/\text{min}$ at any given point, which is a speed generally unattainable by a clustered epithelial cell. Additionally, the final determined ROI must be at least $21\ \mu\text{m}^2$. Cases where these physically reasonable limits are not met are

typically indicative of cell death, division, or multiple cells overlapping. When any of the above criteria fail, the MATLAB interface prompts the user to identify the coordinates of the cell.

For evaluation purposes, the amount of human interaction was recorded. The algorithm was found to analyze an average time-lapse video with $\sim 80\%$ effi-

ciency, relying on human input $\sim 20\%$ of the time. A second tool was developed for qualitatively assessing the algorithm wherein a video of an individual cell was replayed with the center highlighted in order to confirm post hoc that the cell had been tracked properly (Supplemental Videos 1–4). Additionally, a video of wound healing in a sheet of A459 lung carcinoma cells was retrieved from Sandquist *et al.* The cells in this video were analyzed at similar efficiency and migration and migration paths were shown directed into the wound, confirming accuracy of the automated tracking (Supplemental Fig. 2).³⁷

Automated Tracking Identifies Location with Same Precision as Manual Tracking

To validate the algorithm, we applied it to analyze time-lapse images of breast epithelial cell clusters migrating in an applied electric field. The migration of non-transformed MCF-10A cells transduced with a retroviral vector encoding either an empty vector (10A-pLKO) or a vector encoding shRNA targeting E-cadherin (10A-shEcad) was examined. E-cadherin expression of 10A-shEcad cells was previously shown to be 40% of the level expressed by 10A-pLKO cells.²⁷ Three time-lapse videos for each cell type (10A-shEcad, 10A-pLKO) at every applied electric field (0, 0.13, 0.26, 0.51 V/cm) were tracked manually and using the label-free tracking algorithm. Manual tracking was performed twice independently in order to assess variability. The same 1716 cells were tracked in every instance and therefore retained identical initial coordinates.

Positional data from different tracking methods were compared by calculating the distance between the locations of each tracked cell at each time point, as determined by the different methods. These distances, henceforth referred to as residuals, indicate the extent to which the measured trajectories differ between methods (Fig. 2).

We first compared the tracks determined by the algorithm against those determined manually. Three manual datasets were included in the analysis: the tracks obtained in each of the two independent manual trials and the mean of these manual trials. Within ~ 3 frames, corresponding to 15 min of migration time, the residuals increase to a steady-state value. The initial increase to state-state from a residual value of 0 is a result of all of the tracking methods using the same initial coordinates. Soon after the initial few frames, the values of the residuals are maintained at approximately $4\text{--}8\text{ }\mu\text{m}$, regardless of the manually-acquired dataset to which the algorithm was compared. To put this residual in perspective, the diameter of these cells is close to $30\text{ }\mu\text{m}$.

To evaluate how the difference between manual and automated methods compares to the inherent variability of manual tracking itself, we computed the residuals between the two independent manual trials. This residual between the manual tracks behaved similar to those comparing the automated and manual methods, with the two manual trajectories exhibiting a residual of $\sim 4\text{--}8\text{ }\mu\text{m}$. These results demonstrate that the automated algorithm performs within the variability of manual tracking.

E-cadherin Knockdown Reduces Electrotactic Directedness

Having validated the automated algorithm, we expanded our measurements to span 23,565 clustered cells and analyzed this large dataset to elucidate the effect of modulating E-cadherin expression on the directedness of migration within the applied electric field. Both 10A-pLKO and 10A-shEcad cell lines migrated towards the anode of an applied electric field. For cells within epithelial cell clusters, E-cadherin knockdown resulted in weaker directedness within an electric field (Fig. 3). Knockdown of E-cadherin reduced directedness by 16–25% at all electric fields resulting from E-cadherin knockdown, with directedness reaching 0.83 and 0.72 at 0.51 V/cm for 10A-pLKO and 10A-shEcad cells, respectively. Analysis of variance (ANOVA) showed that the reduction in

FIGURE 2. Residuals from comparing tracking methods. The trajectory of the same cells ($n = 1709$) were tracked manually twice in independent sessions and by using the automated algorithm. Residuals are computed as the mean distance between positions of the same cell at each time point tracked by different methods. Comparison of the same cell tracked twice manually is illustrated in blue. Both manual tracks are compared against the automatic detection *via* this algorithm in red. In green, the automatic detection was compared against the mean of the two manual detections. Error bars represent the standard error of the mean.

directedness was statistically significant ($p < 0.0001$). Although a 25% reduction is modest, the large sample size enabled by the automated algorithm provides a high degree of statistical confidence.

Similar to clustered cells, isolated 10A-pLKO cells were more directed than 10A-shEcad although this difference converged at high electric field strengths. ANOVA showed that 10A-pLKO cells in isolation showed an increase in directedness with electric field strength ($p < 0.05$). However, 10A-shEcad cells became significantly directed only at 0.51 V/cm. Consistent with previous findings, isolated cells were approximately half as directed as clustered cells, with directedness of 0.39 and 0.37 for 10A-pLKO and 10A-shEcad cells, respectively, at the highest electric field.

These results show that E-cadherin knockdown diminishes the electrotactic response of clustered MCF-10A cells, albeit not to an extent proportional to the level of reduction in E-cadherin expression. Furthermore, knockdown of E-cadherin inhibits electrotaxis in isolated cells at weak and moderate electric fields, suggesting that E-cadherin regulates electrotactic response in an adhesion-independent manner.

E-cadherin Expression Expedites Electrotactic Reorientation in Isolated Cells

In addition to gauging the role of E-cadherin in orienting cells toward the anode throughout 6 h of exposure to an electric field, we investigated the dynamics of cell alignment. Some cells happen to migrate from the beginning in the direction of the anode. Unlike these cells, another subset of cells are moving initially toward the cathode during the first hour of tracking. We selected and quantified how long it takes for these initially misdirected cells to orient to the anode. To quantify the rate of reorientation, a characteristic time of orientation was calculated by fitting migration paths to Eqs. 3 and 4 (see “Materials and Methods” section).

Cells within epithelial cell clusters took longer to align to the electric field than isolated cells (Fig. 4). Among clustered cells, increasing electric field strength reduced the amount of time taken to reorient within an electric field. The time to reorient decreased more sharply with increasing electric field strength for 10A-shEcad cells than for 10A-pLKO cells. 10A-pLKO cells required a fourfold increase (from 0.13 to 0.51 V/cm) in the magnitude of the applied electric field in order to reorient 2.6-fold more quickly than at 0.13 V/cm. Comparatively, 10A-shEcad cells reoriented 2.4-fold faster after only a twofold increase (from 0.13 to 0.26 V/cm) in the magnitude of the applied electric field.

FIGURE 3. Directedness as a function of applied electric field. Directedness is reported for clustered epithelial cell (dotted lines) and isolated epithelial cells (solid lines) at applied electric fields of 0, 0.13, 0.26, and 0.51 V/cm. Squares indicate 10A-shEcad cells while circles indicate 10A-pLKO cells. Error bars indicate the standard error of the mean. Sample sizes for 10A-pLKO cells at electric fields of 0, 0.13, 0.26, and 0.51 V/cm were 2978, 3653, 2971, and 3032, respectively for clustered cells and 371, 379, 446, and 387, respectively for isolated cells. Sample sizes for 10A-shEcad cells at electric fields of 0, 0.13, 0.26, and 0.51 V/cm were 2728, 2552, 2938, and 2713, respectively for clustered cells and 486, 584, 403, 445, respectively for isolated cells.

Meanwhile, for epithelial cells in isolation, both 10A-pLKO and 10A-shEcad cells reoriented in an electric field within ~ 1 h, faster than when clustered (Fig. 4). The time to reorient did not depend on the electric field strength for isolated cells. Unexpectedly, the time to reorient for 10A-shEcad cells was 61–64% as much as the time need for 10A-pLKO cells (Fig. 4). This difference between cell lines was statistically significant using a t test ($p < 0.05$). These data suggest a role for E-cadherin in regulating electrotaxis independent of its involvement in mediating cell–cell contacts.

E-cadherin Expression Slightly Reduces Persistence but not Speed of Migration

We next investigated the role of E-cadherin in regulating the speed and persistence of migration. Instantaneous speed was estimated as the distance traveled by a cell between frames divided by the length of time between frames. No clear dependence of speed on electric field strength was observed in isolated cells. However, cells in epithelial cell clusters showed a 23–25% increase in speed when exposed to an applied electric field, independent of E-cadherin expression (Supplemental Fig. 3).

Persistence of migrating cells was quantified as the net displacement over the observation time divided by

the total path length taken by the cell.^{7,29} Only cells within epithelial cell clusters display a dependence of persistence on electric field strength (Supplemental Fig. 4). Persistence was observed to increase with electric field ($P < 0.01$ with ANOVA) for both clustered 10A-pLKO and 10A-shEcad cells. While both cell lines shared a baseline persistence close to 0.4, persistence increased more rapidly for cells with reduced E-cadherin expression. However, at the highest electric field, 10A-pLKO cells displayed roughly 10% higher persistence than 10A-shEcad cells. While these differences in persistence are quantitatively modest, the large sample size made feasible by automated tracking confers statistical significance.

The persistence of isolated cells shows no dependence on the strength of the applied electric field, although in the absence of an electric field, 10A-pLKO cells were significantly more persistent than in the presence of an electric field (Supplemental Fig. 4). Taken together, these data show that while E-cadherin has no apparent role in regulating persistence of isolated cells, it has a quantitatively modest role in the persistence of cell migration during collective electro-taxis.

Calculation of Migration Properties is not Dependent on Tracking Method

The above analysis examines migration properties based on trajectories acquired with the automated algorithm. While we established that these trajectories are in close agreement with those identified manually, we sought to validate further that migration metrics (directedness, speed, persistence) calculated from trajectories determined by the automated algorithm are in

accordance with those computed from manually-identified trajectories. To conduct this validation, we returned to the 1716 migration trajectories that were manually identified, twice independently.

Directedness was calculated for these cells and compared between methods (Fig. 5a, d). The directedness of cells was similar for both repetitions of manual tracking (Fig. 5a). Furthermore, the directedness calculated from trajectories determined by the algorithm was in agreement with that calculated from the mean position of manually-tracked cells (Fig. 5d). Out of the 24 videos analyzed by both tracking methods, there was not a single case of statistically significant difference in calculated directedness between the tracking methods.

The persistence and speed of cells tracked by both methods was also compared. The persistence calculated from manually-tracked trajectories correlated well between repetitions (Fig. 5b); the same consistency was observed in calculating migration speed (Fig. 5c). Meanwhile, trajectories identified by the algorithm yielded values of persistence that were 15.2% lower than those calculated from manually-acquired trajectories (Fig. 5e). Speed also exhibits a systematic difference between manual and automated methods: speed calculated from tracks acquired by the algorithm were on average 15.7% greater than those estimated from manually-acquired trajectories (Fig. 5f). This systematic difference is a result of statistical noise in the automated tracking method.

With a mean recorded speed of $0.46 \mu\text{m}/\text{min}$ across all conditions, a 15.7% overestimation in speed corresponds to an error of $0.063 \mu\text{m}/\text{min}$, or $0.31 \mu\text{m}$ per frame, between manual and automated tracking. When comparing the instantaneous speed of each cell

FIGURE 4. Characteristic orientation time to an applied electric field. Cell tracks were filtered for initial migration opposite of the expected steady-state value. This subset of cells is governed primarily *via* stochastic mechanisms. The control case of 0 V/cm does not generate meaningful data in this calculation and was therefore ignored. (a) Orientation time of cells. Large characteristic orientation times are indicative of slow reorientation to an electric field. (b) Separated axes to show orientation time of isolated cells. Error bars represent the 95% confidence interval on calculated parameters. Asterisk indicates statistical significance with $p < .001$ as determined *via* Student's *t* test.

FIGURE 5. Comparison of migration metrics. Speed, persistence, and directedness were calculated for each tracked cell. First, both iterations of manual tracking were compared on scatter plots (a–c) and a linear fit intercepting the origin was determined (solid lines). The linear fits were found to have R-values of 0.737, 0.853, and 0.984 for speed, persistence, and directedness, respectively. The ideal fit (dotted lines) is a 1:1 direct proportionality. Second, the data from the automatically tracked cell was compared against the mean of the two manual tracks (d–f) and a linear fit intercepting the origin was determined (solid lines). The linear fits were found to have R-values of 0.920, 0.928, and 0.977 for speed, persistence, and directedness, respectively.

between methods, this error was calculated as 0.33 to 0.34 μm per frame. Compared to a mean residual of approximately 6 μm (Fig. 2), an error of 0.31 μm is well within the limits of variability in tracking and therefore can be seen as statistical noise. Path-dependent variables, such as speed, are more heavily affected by the precision of tracking at each individual frame than state functions, such as directedness, as evidenced by the overlapping real and ideal fits for directedness (Fig. 5D). This is because the calculated total path length of a cell tracked this way will include the noise at each frame, cumulatively, whereas functions of only initial and final position will only be affected by the noise associated with the final position. Regardless of the overestimation of path length in this method, it should be noted that manual tracking of cells likely underestimates path length because human bias tends to smooth cell movement tracks.

DISCUSSION

In this study, we develop a robust label-free algorithm to track the migration of clustered cells and apply it to elucidate the role of downregulating

E-cadherin expression in the electrotactic collective migration of non-transformed mammary epithelial MCF-10A cells. First, we validate the tracking algorithm by comparing two independent manually-acquired trajectories of 1716 clustered cells with the trajectories determined by the algorithm. This comparison shows that the automated and manual acquisitions of trajectories were in close agreement, with the differences between trajectories being within the limits of user variability. Second, we show that knockdown of E-cadherin diminishes the alignment of cells to an electric field by approximately 20%. Interestingly, this quantitatively subtle and statistically significant effect is observed in both clustered and isolated cells, suggesting an adhesion-independent role for E-cadherin in regulating electrotaxis. Finally, further supporting an adhesion-independent role for E-cadherin, our data show that reducing E-cadherin expression affects the dynamic response to electric fields in both clustered and isolated cells. Reducing E-cadherin expression makes the kinetics of alignment to an electric field more sensitive to the strength of the electric field in clustered cells; meanwhile, isolated cells with reduced E-cadherin expression align to the electric field more

quickly than their counterparts with normal E-cadherin expression levels. These effects of reduced E-cadherin expression on electrotaxis have implications for cancer progression where the downregulation of E-cadherin and electric field-enhanced migration play a role in cancer invasion out of the primary tumor.

A major mode of cancer cell invasion involves collective cell migration, a complex, many-body, dynamic process that requires laborious and time-intensive manual analysis of time-lapse images in order to study with quantitative rigor. Here, we develop, validate, and demonstrate an automated image analysis algorithm that extracts the trajectory of individual cells in a migrating cell cluster. The method is label-free, circumventing the potential pitfalls and additional steps associated with genetically- or chemically-labeling cells and the toxic effects of long-term fluorescent imaging.

Automated image analysis of cell clusters faces at least two significant hurdles. First, given an image with many closely-adjointing cells, individual cells must be identified (the segmentation problem).^{5,26} Second, once identified, each cell in an image must then be correctly linked to itself, and not to some other cell, in the next image of the time series (the correspondence problem).⁴ Here, our algorithm addresses segmentation and correspondence by cropping around an individual cell, processing its trajectory longitudinally over time, and then coming back to repeat the process for the next cell. Briefly, the algorithm begins with the user seeding the algorithm with the initial position of all cells-of-interest. With the initial condition specified, the algorithm crops around each cell and identifies its position sequentially over the time-series of images. Performing segmentation on a sub-image cropped around the immediate neighborhood of an individual cell enhances the robustness of image processing techniques, such as the use of binary filters. A combination of logarithmic contrast enhancement and estimation of the derivative of local image intensity was used to identify cell position from phase-contrast time-lapse images.

To guide the seeding of initial cell positions and to manage circumstances where the algorithm fails to identify a cell, a graphical interface for user input and monitoring of algorithm performance was designed. In circumstances such as changes in the focus of the microscope, condensation within the microscope incubation apparatus, or irregular cell shapes (due to migration in the z-direction over other cells or multi-nucleation as a result of mitotic errors, for example) the user interface prompts for input. Events leading to human input occur roughly 20% of the time during tracking. We therefore estimate approximately a five-fold increase in processing speed. In this study, 23,565 clustered cells were analyzed, and assuming that each

cell trajectory would take one minute to track manually, the algorithm eliminated 320 person-hours of labor. Cell lineage was not included in the algorithm and mitosis within the middle of the time-lapse would result in the track of that cell being discarded. Even with these limitations, the potential applications of this algorithm are widespread, including studying collective migration in wound healing, probing leader cell dynamics in migrating clusters, or analyzing heterogeneous cell-cell interactions in cell sorting or dissemination.

The trajectories identified by the automated algorithm are in close agreement with manual tracking, with the disparity between the two methods falling within the limits of human variability. Furthermore, cell migration properties, including directedness, speed and persistence, calculated from trajectories acquired using the automated algorithm are highly correlated to those calculated from manually-determined trajectories. In particular, directedness is measured with little discrepancy between automated and manual methods, indicating that both tracking methods perform equivalently in detecting changes in the direction of cell migration. Meanwhile, speed and persistence quantified by manual versus automated methods exhibit systematic discrepancies, with the value for speed and persistence being systematically lower and higher, respectively, for manual tracking compared to automated tracking. Because the speed and persistence are directly and inversely proportional, respectively, to the total observed path length of the cell track, we can conclude that this systematic error caused by statistical noise in the cell positions determined by the algorithm which results in a greater observed total path length. Conversely, manual tracking methods are likely to underestimate instantaneous displacement due to inadvertent track smoothing. This smoothing effect is due in part to the design of manual tracking user interfaces, such as the ImageJ tracking interface, where there is a tendency to lag behind the cell being tracked since the cursor position always remains at the coordinates of the cell in the previous frame.

While user behavior that leads to trajectory smoothing is difficult to measure and demonstrate unequivocally, it is important to quantify the magnitude of the systematic error and determine to what extent it may affect the analysis of cell migration. The systematic difference in cell speed estimated by manual vs automated methods is approximately 15.7%, corresponding to an error of 0.33–0.34 μm per frame. For comparison, the residual in cell coordinates estimated by two independent iterations of manual tracking is approximately 8 μm . Therefore, we conclude that the systematic error is much smaller than the variability inherent to manual tracking. As such, the automated

approach provides as accurate an assessment of cell migration properties as a manual approach, while offering the aforementioned benefits of high throughput data analysis and label-free imaging.

Applying the automated image analysis algorithm, we analyze the migration of 23,565 clustered MCF-10A cells and show that downregulating E-cadherin expression by approximately 60% affects both the kinetics and steady-state alignment of collective cell migration to an external electric field. Clustered cell migration aligns more quickly to stronger electric fields. This sensitivity of alignment kinetics to the strength of the electric field increases two-fold in cells with reduced E-cadherin expression when compared to control cells with normal levels of E-cadherin expression. Previously, we have shown that clustered cells, while slower than isolated cells to reorient to an electric field, accelerate reorientation at strong electric fields. We postulate that the breakdown of cell–cell contacts may be responsible for the accelerated response only after a critical electric field, sufficient to overwhelm contact inhibition of locomotion, is applied. With weakened cell–cell contacts, the threshold for this accelerated reorientation is reduced in 10A-shEcad cells to a value between 0.13 and 0.26 V/cm compared to a value between 0.26 and 0.51 V/cm for 10A-pLKO cells. Meanwhile, steady-state directedness is reduced by approximately 20% in cells with reduced E-cadherin expression compared to control cells.

While these data demonstrate a role for E-cadherin in regulating electrotaxis of clustered mammary epithelial cells, our findings also suggest a role for E-cadherin-independent mechanisms in collective electrotaxis. Reducing E-cadherin expression diminishes the directedness of clustered cells by approximately 20%, a level that still remains significantly greater than that exhibited by isolated cells. We do not rule out that reducing E-cadherin expression even further may ultimately diminish directedness of clustered cells to a level that matches isolated cells. In this regard, using alternate shRNA constructs to target E-cadherin may yield different levels of E-cadherin knockdown, offering a means to determine the sensitivity of electrotaxis to changes in E-cadherin expression level and providing a way to further corroborate the partial role of E-cadherin. Meanwhile, based on the present dataset, the disproportionate 20% reduction in electrotaxis in response to a 40% downregulation in E-cadherin expression leaves open the possibility that other aspects of cell clustering render collective movement significantly more responsive to electric fields than isolated cells. These additional aspects of a clustered microenvironment include cell crowding as well as other mediators of cell–cell interactions, including gap and tight junctions, albeit the latter is less likely to be

relevant in MCF-10A cells that are known to express low levels of ZO-1 and form poor tight junctions.¹³

In contrast to our findings in mammary epithelial cells, research in MDCK cells implicate E-cadherin as the chief regulator of collective electrotaxis.²² Inhibition of E-cadherin by blocking with DECMA-1 or depleting extracellular Ca^{2+} rendered clustered MDCK cells incapable of orienting toward an electric field. Several factors may contribute to the discrepancy between the MDCK study and our results.²² First, to what extent blocking with DECMA-1 or depleting extracellular Ca^{2+} diminishes E-cadherin functionality in the MDCK study and how that compares to the 40% reduction in E-cadherin expression in this study is difficult to assess. Furthermore, some of the observed differences may be attributable to differences in cell types. Unlike isolated MCF-10A cells, isolated MDCK cells fail to exhibit electrotaxis. Thus, in MDCK cells, cell clustering is a requirement to undertake electrotaxis, and therefore, compared to MCF-10A cells, MDCK cells may be more reliant upon E-cadherin-mediated cell adhesion in order to direct their migration within an electric field. Quantitative differences notwithstanding, both our results with mammary epithelial cells and those with MDCK cells agree on a role for E-cadherin in collective electrotaxis.

Because isolated mammary epithelial cells exhibit electrotaxis, this cell system offered an opportunity to test whether E-cadherin has an adhesion-independent role in electrotaxis. Unexpectedly, at low and moderate electric field strengths, isolated cells with reduced E-cadherin expression exhibit approximately 20% lower steady-state directedness when compared to control cells. Furthermore, reduced E-cadherin expression enhanced the kinetics of orienting the migration of isolated cells to the electric field. These observations demonstrate that E-cadherin has adhesion-independent effects on the electrotaxis of mammary epithelial MCF-10A cells. E-cadherin may mediate adhesion-independent effects *via* several possible mechanisms, including downstream intracellular signaling through β -catenin and p120-catenin.³² We have shown previously that EGF induces β -catenin signaling in MCF-10A cells seeded at a sub-confluent density.¹⁶ E-cadherin also associates with and modulates the functionality of other cell surface receptors, including the EGF receptor,^{17,47} which has been implicated in regulating electrotaxis.^{34,43,48}

In summary, we show that E-cadherin, likely working in concert with other aspects of cell–cell interactions, plays a role in the superior electrotactic ability of clustered cells compared to isolated counterparts. Interestingly, E-cadherin also plays an adhesion-independent role in the electrotaxis of isolated cells. Since downregulation of E-cadherin is a common

event during cancer progression,^{30,41} our results have implications for how cells with reduced E-cadherin expression may invade, either collectively or individually, through tumor microenvironments in which electric potentials are known to be prevalent.²³

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (doi: [10.1007/s12195-016-0471-6](https://doi.org/10.1007/s12195-016-0471-6)) contains supplementary material, which is available to authorized users.

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CONFLICT OF INTEREST

Mark L. Lalli, Brooke Wojeski, and Anand R. Asthagiri declare that they have no conflict of interest.

ETHICAL STANDARDS

No human or animal studies were carried out by the authors for this article.

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