THE INFLUENCE OF THE BIOPHYSICAL ENVIRONMENT AND CELL-CELL INTERACTIONS ON EPITHELIAL CELL ELECTROTAXIS

A Dissertation Presented

By

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To

The Department of Chemical Engineering

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In the Field of

Chemical Engineering

Northeastern University
Boston, Massachusetts

July 20, 2016
Abstract

Cell migration plays a pivotal role during tissue growth and development, wound repair, and progression of metastatic disease. Mechanical, chemical, and electrical cues all provide stimuli to direct cell migration. Electric fields as stimuli for directed cell migration, known as electrotaxis, have attracted attention in recent years due to their ability to override migratory cues from other types of stimuli. The use of electrotaxis has promising application in wound repair, where it is being utilized to treat chronic non-healing wounds such as ulcers. In this study, we sought to investigate the interactions between electric fields and intercellular connections on the electrotaxis of epithelial cells.

We found that the non-transformed mammary epithelial cell line, MCF-10A, cells migrate toward the anode of an applied electric field. Although cells in isolation will display electrotaxis, they require an electric field at or above a threshold of 0.26 V/cm in order to migrate toward the anode. Increasing the strength of the stimulating field increased the degree to which the cells migrate toward the anode. However, when adjacent to other cells in a cell cluster, they not only migrate toward the anode with more directed paths, but also require no threshold of electric field to begin migration. Analysis of these migration paths indicated that being in the clustered cell state inhibited reorientation for migration.

To investigate the non-geometric effects of cell clustering, expression of the cell adhesion protein, E-cadherin, was reduced in MCF-10A cells by 60%, since E-cadherin is a major player in epithelial intercellular interactions. Cells within clusters migrated toward the anode of an applied electric field even when E-cadherin expression was reduced. However, the degree to which movement was aligned with the electric field was
reduced by 16% compared to non-transformed cells. Isolated cells with E-cadherin knockdown were also found to reorient to the electric field more quickly and, while they were equally aligned at 0.51 V/cm, they were significantly less directed at electric fields below this.

Since strong electrotaxis is often associated with metastatic potential of cancer cells, the electrotaxis of highly invasive MDA-MB-231 breast cancer cells was investigated. To spatially confine cells, the electrotactic chamber was first filled with a confluent layer of noninvasive breast epithelial cells. E-cadherin expression was perturbed in this system as well, with additional E-cadherin expression reducing overall electrotaxis of invasive cells. This is consistent with E-cadherin being a tumor suppressor and prevent invasion of cells. In a modified transwell assay, invasion was lost with increased E-cadherin expression but rescued upon exposure to a weak electric field. These results indicate that both cell-cell interaction and spatial confinement associated with the tumor microenvironment play independent roles in modulating electrotaxis.

Overall, the data presented herein reveals how electric fields and the cellular environment interact with epithelial cells to generate a migratory response. Our work elucidates the operational space for tissue engineering applications using electric fields to spatially direct cells. We also show that the tumor microenvironment may act synergistically with bioelectric fields to promote metastasis. We present several new platforms that can be used to investigate molecular perturbations, which we expect will lead to the development of new chemical therapeutics which act upon electrotactic pathways.
Acknowledgements

This work could not have happened without the support and guidance of my advisor, Professor Anand Asthagiri. His tolerance of my fixation on electricity and his trust that I could bring this project to fruition allowed us to develop this electrotaxis project in our lab. Sometimes, speaking with Anand can be intimidating, not because of his personality but rather because of the level of thought put into every word he says. Without the thought that Anand put into our conversations, I would not have been able to have completed the goals we laid out in front of me.

The enduring mutual support from my labmates, Dan Milano, Robert Natividad, and Emily Gong will always be appreciated. For four years, they have been a second family to me, and, although I admit sadness at seeing our parting of ways, I am excited to see the things we will all accomplish. The extensive work done by my undergrads, Brooke Wojeski and Hannah Orzechowski, was invaluable. I also thank Rob Eagan, for his ever immediate responses when it comes to help, as well as David McKee and Scott McNamara at the Kostas Center, for their support and patience.

Lastly, I’d like to thank my family and friends for their enduring support of me and for being my inspiration to achieve. My family is responsible for my continued stay in academia even when conditions were less than ideal. I’d like to thank my friends who understand that “I can’t, I have lab” is a valid reason for not seeing them rather than a flimsy excuse. My roommates, Lisa and Jessica, have been especially wonderful to me during what has been the hardest part of this process so I’d like to thank them for forced study breaks and glasses of wine. And lastly, the unconditional love and encouragement from Kassi has kept me going even at the hardest times. Thank you.

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Dedication

When I was a junior in high school, my father was teaching me to drive a car. We would drive to school together each day while I had my learner’s permit, then he would take the car to work. One day, I was turning right onto South Main Street. Another car, turning left from the opposite direction, cut me off. “I should have let him hit me,” I said. “It would have been his fault.”

My father replied, “Yes, but you would have been late to school.”

I wasn’t late to school.

This thesis is dedicated to my mother and father, for instilling in me the motivation to always work toward my own education and to become the best person that I can be. Without them, none of this would be possible.
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1. Introduction

Bioelectricity has been known of since the time of the ancient Egyptians, who described the paralyzing shocks delivered by the electric eel [1]. The ancient Greeks are known to have discovered that static electricity applied to muscles could cause twitching. The use of amber, called ἥλεκτρον in Greek, to generate static electricity gave rise to the name “electron” in English. However, it was just over three decades ago that electric fields arising from wounded tissue were discovered [2]. The term “skin battery” was used to describe this phenomenon and, although the possibility of its role in wound healing was discussed, it was not until almost a decade later that direct observation showed human cells migrating in physiologically relevant electric fields [3]. While various mechanisms have been proposed [4]–[8], a definitive pathway has not been identified. While the underlying mechanisms are unclear, electric fields show promise in directing cell migration in a highly controllable manner in applications such as tissue engineering, especially with respect to neural systems [9], [10].

Remarkably, electric fields are such a potent director of cell migration that, often, other stimuli are overridden when an electric field is present [11]. In fact, not only have electric fields been used therapeutically for wound closure [12], but application of an opposing electric field to the direction which would arise physiologically results in further wound opening [11].

Despite the potency of directional guidance provided by electric fields, they remain the least utilized and studied guidance stimulus. Based on a simple literature search, the role of chemical gradients is studied nearly 300 times more frequently than the effect of electric fields for directing cell migration. The physiological relevance of
chemical gradients is well understood and has been documented for decades [13]. Molecular factors such as transforming growth factor beta (TGF-β) or vascular epidermal growth factor (VEGF) are known to be important to the physiological processes such as the immune response [14]–[16].

Perhaps to a similar degree as chemical gradients, electric fields are nearly ubiquitous in physiological and pathophysiological contexts. Electric fields have been measured within ductal systems such as the breast [17] and the prostate [18]. These electric potentials exist on the order of tens of millivolts but, at the length scale of the breast and prostate ducts, can produce electric fields on the order of 1-10 V/cm. Even stronger electric potentials up to hundreds of millivolts can be detected as a result of the electrokinetic flow of blood within human vasculature [19], [20]. The tumor microenvironment (TMEN) is electrically active as a result of accumulation of negative surface charge during rapid cell growth and phenotypic changes during cancer progression [21]. Recent evidence suggests that even the human gut microbiome may be exoelectrogenic, using electric fields to recruit immune cells [22].

Considering the prevalence of electric fields in physiological settings, it is not surprising that many cells across a wide range of phenotypes migrate directionally within an electric field, known as electrotaxis. Among the cells which undergo electrotaxis are epithelial cells [23], [24], fibroblasts [25], and lymphocytes [26], [27]. Even non-mammalian cells such as amoeba [4] or whole organisms such as the nematode Caenorhabditis elegans [28], [29] are known to migrate directionally with an electric field.
Furthermore, electrotaxis is emerging as an important mechanism in metastasis. Several metastatic cancer cells such as breast, lung, and prostate cancer cells, exhibit electrotaxis [7], [30]–[32]. In fact, strong electrotaxis has been correlated with a high metastatic potential [17]. The metastatic cascade involves five parts, 1) development of an invasive phenotype within a primary tumor, 2) local invasion resulting in entering the blood stream, 3) survival in suspension through the blood, 4) extravasation into foreign tissue, and 5) survival and proliferation resulting in a secondary tumor [33]. Electric fields are likely to be present during step 2, where they could serve as a guidance cue aiding the migration of invasive cells away from the primary tumor and toward the blood stream. Chemotherapeutic agents typically work by preventing proliferation, such as vincristine [34] and doxorubicin [35]. Some chemotherapeutics, such as asparaginase, starve cancer cells of nutrients which they cannot synthesize themselves, resulting in cell death at any of the 5 steps of metastatic cascade [36]. Understanding the relationship between metastatic potential and strong electrotaxis would help develop therapeutics that act specifically on step 2 of the metastatic cascade and prevent formation of secondary tumors.

Incorporation of physiologically relevant platforms to quantitatively measure and analyze electrotaxis is required for progression of the field. This challenge has been addressed herein by fabricating new assays which allow the different aspects of electrotactic cell migration to be analyzed. Namely, the role of intercellular interactions and influence of spatial confinement in a heterogeneous environment were analyzed separately. Not only does understanding of electrotaxis present a unique opportunity for
tissue engineering applications, but also has significant potential in developing novel pharmacological compounds.
2. Critical Literature Review

Electrotaxis is a significant cellular behavior that is broadly relevant to many physiological processes, and yet is relatively poorly understood. This review will cover electrotaxis in many settings. Specifically, cell migration in general and in the context of single and collective migration is addressed. Physiological contexts of electrotaxis, such as in wound healing, are presented. Lastly, the operating space of the current state-of-the-art in investigating directed cell migration is discussed. The ultimate goal of this review is to indicate the gaps in the current body of knowledge surrounding electrotaxis and to show how the work presented in this thesis meets the current challenges.
2.1 Cell Migration in Normal Physiology

Cell migration is a highly dynamic process in which cells, either individually or collectively, traverse the extracellular matrix (ECM). A variety of processes including organogenesis, wound healing, immune response, or in the case of cancer, metastasis, require cell migration. In this process, cells rely on a system of microtubules and a network of actin filaments, known as the cytoskeleton. In many cases, cell migration involves the extension of lamellapodia, which are protrusions of the plasma membrane driven by the cytoskeleton, toward the direction of movement and the retraction of membrane at the opposite edge of the cell [37]. Without an external stimulus mobile cells engage in a “random walk” where they undergo bouts of migration in a given direction before reorienting and migrating in another direction at random [38]. In the presence of certain external, graded stimuli, cells direct themselves and move in a specific direction. This type of directed migration includes movement up or down a gradient in concentration of a chemical cue (chemotaxis) [39], [40], adsorbed ligands on the ECM (haptotaxis) [41], [42], substrate stiffness (durotaxis) [43], and electric potential (electrotaxis) [44]. These directed modes of migration are typically associated with a polarization of the cell body in the direction of travel. A frequently used measure of a cell’s polarity is given as the axis defined by the locations of the nucleus and the microtubule organizing center (MTOC) [45].

2.1.1 Single Cell Migration

A common mode of migration for adherent, single cells depends on several events occurring. First, a cell must generate protrusions at the leading edge, typically by producing actin filaments. Actin filaments are polymerized from monomeric actin in a
process known as “treadmilling” [46], [47]. The process earns this name because polymerization and depolymerization kinetics favor growth in a single direction while favoring disassembly at the rearward “barbed” end [46]. As actin monomers are added to the polymer, they exert forces on the membrane, temporarily protruding it and pushing preexisting actin units toward the rear of the chain. This new area of the cell then must make adhesions with the underlying ECM while adhesions on the rear end of the cell must come undone [48]. The disassembly of rearward adhesions is typically regarded as the rate limiting step [49]. This process can be modeled from a chemically kinetic standpoint as the formation of adhesions and retraction of adhesions occur with different rates at varying locations along the cell body. Translocation of the cell body is then possible using acto-myosin generated contractility which pulls the cytoskeleton toward the newly formed adhesions and adhesive complexes in the front of the cell. Below, Figure 1 shows the redistribution of actin filaments extending the membrane of a cell to one side and then proceeding to migrate in that direction.

![Figure 1](image)

**Figure 1.** Actin redistribution and migration of bovine aortic endothelial cells in response to an electric field [102].

Throughout this process, organelle position is critical and the migrating cell is typically observed to orient itself with some degree of polarity. In general terms, polarity is defined as an asymmetry of the cell in its shape or in the distribution of proteins and
molecules within it [50]. Migration, surface adhesion, and cell-cell contacts all induce polarity within a cell [45], [51]–[53].

For a single adherent cell, the degree to which the cell adheres to the substrate plays a significant role in its ability to migrate. For instance, cell speed has a parabolic relationship with cell-ECM adhesion where cell motility is inhibited at low and high levels of adhesion and maximized at an optimal level between them. At low levels of adhesion, cells do not spread onto a substrate very well and cannot form the adhesions necessary for acto-myosin contractility to exert forces on in order to propel the cell. On the other hand, when adhesion levels are too high, cells are incapable of disassembling preexisting adhesive complexes located toward rear end of the cell body, thereby inhibiting movement in any particular direction [54]–[56].

Cells respond to a number of soluble factors such as proteins and small molecules, some of which attract cells via chemotaxis while others repel cells. It is well known that epidermal growth factor (EGF) causes chemotaxis when cells are exposed to a gradient in concentration and chemokinesis when cells are exposed to a flat level in its concentration [57]–[59]. Chemokines are typically sensed by the cell by coupling with a heterotrimeric G protein complex which then dissociates into its component parts and activates a signal transduction pathway within the cell.

Single cell motility assays range in scope depending on the level of measurement desired. A conventional assay is the use of the Boyden chamber in which cells are seeded upon a filter with known pore size which separates cell culture media in the upper volume and media with the chemical of interest in the lower volume [60]. This is also known as a transwell assay because the fraction of cells which move through the filter is
recorded as the chemotactic metric. Similarly, the Dunn and Zigmond chambers utilize a bridge connecting concentric circular or parallel rectangular wells, respectively, and count the number of cells which move across the boundary from one well to the other [61], [62].

The development of microfluidic devices has opened many possibilities for generating highly specific and stable chemical [41], electrical [63], or stiffness gradients [64]. In such customizable setups, live imaging and tracking of individual cells is possible and the tracks of these cells are collected in real time. The most notable difference between such a microfluidic device and a Dunn or Zigmond chamber, for instance, is that the chemical gradient of a Zigmond chamber is slowly eliminated as diffusion equalizes concentrations on either side of the bridge. Moreover, microfluidic devices offer the ability to expose the cells of interest to more than a single stimulus by combining different tools.

2.1.2 Collective Cell Migration

Collective cell migration is a necessary function of many cell types for such process as organogenesis [65], angiogenesis [66], and wound healing [67] and is distinguished by the preservation of intercellular bonds [68]. In order for collective migration to occur, a highly regulated balance of external cues and intercellular signaling must occur. The driving forces for collective migration can range from the availability of space to local haptotactic cues and global chemotactic or electrotactic signals and can be mediated by sheer stress and contact inhibition. Often, matrix remodeling precedes collective migration, as in the case of tumor development [69].
Collective migration involves complex signaling between cells in order to coordinate movement in a single direction [70]. This model proposes that collective migration in response to external cues may be a method by which biological systems eliminate environmental noise and other minor fluctuations. Maps of the traction forces exerted by cells on substrates have shown that individual cells within a cluster are not entirely self-propelled and they are directed by the cell in front of them [71]. Up to the first three rows of cells migrating to the leading edge of a wound were observed in this case to be transmitting forces to the cells immediately behind them, which although may be insignificant in actually moving the cells, it is most likely sufficient to trigger a mechanically transduced signal in the neighboring cell.

During times of cell-cell contact, forces perpendicular to the contact surface maintain tension between the cells. It was found in canine kidney epithelial cells (MDCK) that the forces exerted at this interface were constant around 100 nN and independent of the area of interface [72]. However, the forces are only balanced if there is no net migration. In cases where sheets of cells are permitted to migrate onto new surfaces, force coordination between cells up to six cell lengths away [73]. Trails of single cells exhibited inchworm-like cycles of extension and contraction while large regions in the interior of an advance sheet developed vortices of coordinated forces. Ng et al. found that correlation of velocity vectors tapered off after ~100 microns [74]. Using automated tracking of cells, “streams” of coordinated movement two cells wide were discovered [75]. Although directed migration of sheets and clusters of cells is important in normal tissue function and development, when left unregulated it can lead to a diseased state.
Interesting, collective migration of cells within an applied electric field shows an enhanced response to the stimulus. Li et al. describes the migration of MDCK canine epithelial cells under an electric field [76]. While cells in isolation were not observed to undergo migration within an electric field, cells within an epithelial layer did. A possible difference between cells in isolation and confluent cells are the presence of gap junctions, which allow ion diffusion and electromigration between the cells. However, they report that blockading gap junctions was not sufficient to prevent electric field directed migration in this system. They did find, however, that chemical inhibition of the surface receptor E-cadherin prevented electrotaxis of the epithelial layer.

2.1.3 Metastasis

In over 90% of cancer related deaths, metastasis was found to be involved [77]. Although tumors are highly unique between patients, certain traits of the tumor microenvironment (TMEN) are often conserved. Among these traits are low oxygen content and limited space [78]. Loss of gene expression for proteins which help repair DNA damage are generally an early step in cancer progression, as they permit the accumulation of further mutations [79]. In this environment, transformed cells compete in a Darwinian selection process and continue to genetically diversify, which leads to certain phenotypes that are common for many cancers, including, for example, a much higher reliance on anaerobic metabolism [80]. In fact, it has been reported that cancer cells utilize glycolysis for 50 to 70% of ATP generation [81], [82].

This process of mutation accumulation and proliferation leads to a highly heterogeneous genotypic and phenotypic collection of cells. Metastasis begins to become favored in the progression of the disease because it affords cancerous cells access to new
locations and abundant resources. However, in order to complete metastasis, cancer cells must be able to 1) exit the primary tumor, 2) enter the circulatory or lymphatic system, 3) initiate invasion of local tissue in a new area, and 4) proliferate to produce a new tumor. Although one might suspect that a culmination of mutations is required for a cell to acquire the traits necessary to complete every stage in this process, recent work by Carey has shown that invasive leader cells (human breast adenocarcinoma cell line MDA-MB-231) were able to induce invasion in noninvasive human epithelial breast cells (MCF-10A cell line) [69]. This work elucidates not only collective migration in a cancer context by showing that metastatic cells do not operate independently, but also shows that the heterogeneity of the tumor environment provides a set of phenotypes which, through biochemical and biomechanical signaling, are capable of facilitating in the migration of metastatic cells away from the primary tumor. Moreover, the tumor environment is closely associated with other, non-cancerous cells which are involved in matrix remodeling and angiogenesis [83].

Angiogenesis is itself a type of collective migration in which endothelial cells respond to chemoattractants like VEGF as well as ligands adherent to the ECM in order to create new or extending existing blood vessels toward the source of the signal (reviewed in [66]). This process is critical during tumor progression, where cancer cells trigger the formation of new blood vessels by secreting growth factors [84]. Additionally, at secondary tumor sites, invasive cells need to be able to begin the vascularization of tissue or the resultant tumors will be small, clustered at the vessel surface, and have high levels of apoptosis [85].
2.1.4 E-Cadherin

E-cadherin is a membrane bound protein which is used to create and maintain cell-cell adhesions. Physiologically, E-cadherin plays a significant role in maintaining the organization associated with collective migration [67]. Structurally, it is composed of five repeating units which extend beyond the cell membrane, one unit within the cytosol, and one unit within the cell membrane itself (Fig. 2) [86]. Within the cytosol, E-cadherin interacts with catenins, which regulate the organization of the actin cytoskeleton at the membrane [76]. Outside of the cell, the extracellular component, which requires calcium to form bonds, can either undergo homophilic interaction by docking the “acceptor pocket” and the “adhesion arm” together, or heterophilic interaction by docking to an E-

Figure 2. Structure of E-cadherin. Adapted from [228].
cadherin molecule bound to another cell [87]. While the “acceptor pocket” and “adhesion arm” are charged, there doesn’t appear to be a net molecular charge associated with E-cadherin [88].

E-cadherin is a known tumor suppressor and the loss of E-cadherin expression is often associated with metastatic progression [89]. The loss of E-cadherin is thought to be partially responsible for the increased invasive behavior associated with tumor cells [90]. During cell migration, including metastasis, movement of surface bound molecules may occur as a result of external stimuli [91]. E-cadherin is closely associated with the actomyosin network, which is composed of fibers extending all throughout the cell interior [92]. Movement of E-cadherin within this network is tethered by an effective drag force up to 30 pN/μm and it is possible that such a drag force is acting on other membrane bound proteins [93]. Beyond mediating cell-cell interactions, E-cadherin may play a role in regulating cell migration via this drag force generated from the locally organized cytoskeleton.

2.2 Electrotaxis

Electrotaxis is the process of directed cell movement in either direction parallel to an electric field, i.e. motion directed toward the anode or cathode of an electric field. The fact that cells respond to electric fields is well known despite electrotaxis being far less studied than chemotaxis. The study of electrotaxis was made more prevalent by the work done by Song et al. in designing a simple to use electrotactic device, which has been used predominantly since [63].

The presence of electric fields within the body has been proposed as an explanation for the existence of electrotaxis. For instance, the breast epithelial lining has
a typical TEP of 30 mV and human skin has a TEP of 10 – 60 mV [2], [17]. When this epithelial layer is broken, ions are free to move through the wound and generate an ionic current. These endogenous electric fields have a wide variety of effects on cells which may or may not involve migration. Wang et al. shows that electric currents were capable of arresting the cell cycle in corneal lens epithelial cells and that endogenously generated currents are lost upon removal of the lens during cataract surgery, leading to increased cell proliferation resulting in disease [94].

Many cell types display electrotaxis including various types of epithelial cells as well as breast, prostate, and lung cancer cells [44], [95]–[100]. The mechanism by which cells perceive and respond to an electric field is not well understood. Work has been done to show that both voltage gated sodium channels and ErbB1 expression affect the process of electrotaxis in breast cancer cells [57], [101]. It is proposed that calcium movement in the presence of an electric field may generate an internal gradient which in and of itself can trigger voltage gate sodium channels (VGSCs), causing a signal cascade [97]. It has also been shown that actin polymerizes directionally in the presence of an applied electric field which may indicate that all cell types should display some type of electrotactic behavior regardless of its physiological application [102]. Interestingly, Dictyostelium discoideum shows an electrotactic response proportional to the square of the field strength which may indicate that voltage sensitive enzymes are responsible for sensing the electric field [103].

Application of an external electric field was not only found to be a stimulus for directed movement, but it was found to override counter stimuli such as the opening of wound [104]. In that work, wound healing of a sheet of Chinese hamster ovary (CHO)
cells was encouraged and quickened by the application of an electric field with the cathode located within the wound. Wound healing was also stopped by applying the field in reverse and the wound was actually opened further by increasing the voltage beyond the level required to halt wound healing.

2.2.1 Wound Healing

As with other modes of collective migration, wound healing is marked by correlated translocation of cells, preservation of cell-cell contacts, cell body polarization, and cell spreading being due to movement as opposed to proliferation. The degree to which cells correlate with others within the same sheet has been shown to be both a function of location from the leading edge of the wound and the stiffness of the substrate [74], which implies that mechanotransduction may be a regulator of collective movement.

During wound healing, as with single cell migration, cell polarization is a crucial step. Recent work has shown that electrical fields have the ability to enhance or inhibit sheet wound healing by overriding other cues to which the cells would respond [24]. There is a physiological basis for such behavior founded in the field of bioelectricity since the discovery of ionic currents originating in wounded tissue in the 17th Century by Carlo Matteucci. Similar to a chemotactic cue which may be released by injured cells upon death or released as part of the immune response, this ionic current can be sensed by cells in the area of wounding, possibly through PI3K, PTEN, ERK, and Rac signaling (reviewed in [11]). This signal overrides other cues that the cells may be responding to, which may indicate the importance of these endogenous electric fields within our bodies.
2.2.2 Electrotaxis in Cancer

The Zhao group has done much work analyzing the highly metastatic MDA-MB-231 breast cancer line as well as the weakly invasive MCF-7 breast cancer line, showing that MDA-MB-231 cells migrate toward the anode while MCF-7s migrate, with very low directedness, cathodally [97]. The response of the MDA-MB-231 cell line to the electric field was dependent on field strength, with directionality peaking at 3 V/cm. This corresponds well to the tumor environment, similar to a wound, generating an endogenous electric field which may act as a stimulus for migration away from the tumor [102]. Because of the heterogeneity of cancer cells within a single tumor, cells capable of electrotaxis in response to this endogenous electric field may be evolutionarily selected for metastasis. Of interest is that the resting membrane potential of various breast tissue cell lines correlated inversely with the degree of metastatic potential, with non-transformed epithelial cells having a resting potential of nearly -50 mV while highly metastatic cells had a resting potential of only -20 mV [101].

This transmembrane potential is tied closely with electrotaxis, as noted by Gao in which he reported that by altering the pH of the medium to either basic or acidic conditions, electrotaxis could be inhibited in Dictyostelium cells [5]. He also found that by increasing the concentration of potassium ions, both chemotaxis and electrotaxis were inhibited, although electrotaxis was inhibited to a much larger degree. Lastly, by electroporating the cell membranes using high voltage pulsed electric fields, he reports that electrotactic behavior was eliminated entirely. This model system is used because of the ability of Dictyostelium to migrate as effectively under a wide range of membrane potentials. These data support the link between having a polarized membrane potential
and electrotaxis, although it is not clear whether or not simply having a membrane potential is sufficient to allow electrotactic behavior or if having localized membrane potentials is a factor.

It has been known since the 1980s, with the development of voltage sensitive dyes [105], [106], that membrane potential is not a single, homogenous entity associated with a cell [107]. It can be affected locally by the presence of lipid rafts within the cell membrane [108], gap junctions between neighboring cells [109], or the possibly the presence of an externally applied electric field, itself [110]. This last effect is caused by the immediate depolarization of the cathode facing side of the cell membrane and a hyperpolarization of the anode facing side if the cell membrane is permeable to ion flow. In this case, a calcium wave has been observed to move from the anode facing side to the cathode facing side over the course of less than 10 seconds [111]. It is possible that the cells may detect this ion flow within their own cytoplasm, although it is only induced through pulses, or at the very onset of an electric field. A material balance on calcium indicates that the steady state ion concentration must remain constant after an initial wave or calcium ions would build up on the side facing the cathode. This effect may be counterbalanced by the activation of voltage gated calcium and sodium channels.

2.2.3 Signaling in Electrotaxis

Unlike chemotaxis, the signaling pathway for electrotaxis does not rely on G protein coupled receptors although Gβ null mutants in Dictyostelium discoideum ameba did show suppressed electrotaxis, indicating nonessential involvement from the Gβ subunit [4]. In this same cell system, PI3Ks and cGMP were found to direct signaling toward the cathode while the N-terminal of sGC directed signaling toward the anode
These multiple signaling pathways can be knocked out relative to each other in order to switch the electrotactic response of single cells. Moreover, gc-null and gbpC-null mutants displayed directed movement only for a short amount of time before returning to random migration, which may imply an integral based control mechanism. In human dermal fibroblasts under an external electric field, the TGF-β signaling pathway was found to be upregulated as well as anti-apoptotic genes and the MAPK pathway [113]. In keratinocytes, β2-adrenergic receptors were found to regulate migration speed in a cAMP-independent mode while also regulating electrotaxis in a cAMP-dependent mode [114]. In good agreement with those results, the Chao group also decoupled cell motility and directionality when they reported that integrin polarized in the cell membrane in response to applied electric fields and controlled the directionality of the cell via RhoA [96]. Applied electric fields also alter gene expressions, as noted by an up-regulation of ACVR1B, FYN, and CTTN as well as a down-regulation of PTEN in CL1-5 cells [115]. This further connects electrotaxis with well-known cell migration and cancer related genes.

Electrotaxis may, in part, be a specific subset of chemotaxis, rather than an entirely separate mode of migration. The electromigration of surface receptors is linked to the electrotaxis of keratocytes [116]. EGFR, for example, has been shown to migrate towards the cathode-facing side of a cell with an applied electric field [117] and has been extensively shown to be involved with chemotaxis of epithelial cells [57]–[59]. Additionally, many signaling pathways associated with chemotaxis are conserved in electrotaxis. For instance, inhibition of PI3K inhibits electrotaxis in neural stem cells
Inhibiting VEGF, ERK, and the Rho/ROCK pathways also reduces electrotaxis [118], [119], [120].

### 2.2.4 The Physiological Electric Field

Electric fields arise in many ways. Probably the most commonly observed electric fields are those associated with power generation and are created by passing a magnet past a coil of metal. They can also be created by harnessing the electron transport during redox reactions, namely metal-metal sulfate or phosphate substitution in chemical batteries or hydrogen combustion or halination within fuel cells. However, electric fields are not limited to industrial production. Of the four electrokinetic phenomena, streaming potential and sedimentation potential result in generation of electric fields [121]. Streaming potential occurs when a liquid is forced to move against a charged surface, for example blood being pumped through the vascular system. Values of 100-400 mV have been recorded at the interface between blood and endothelium, characteristic of the zeta-potential of the electric double layer [19]. This potential has been measured over 20 years ago with strengths of 1.35 mV/cm [122]. Figure 2 below shows the origin of the transepithelial potential and the effect of wounding.

![Figure 2](image2.png)

**Figure 3.** Electric fields originating at a wound site. Adapted from [11].
The field strength of such wounds has been experimentally determined in a number of systems. In guinea pig skin wounds and bovine corneal wounds, the electric field generated was recorded to be 1-2 V/cm and .4 V/cm, respectively [123], [124]. Similarly, human and mouse skin also show electric fields in the range of ~1.2 to 1.8 V/cm [125]. In vitro studies have shown voltage gradients of up to 5 V/cm across the epithelial lining of the rat prostate gland [126].

2.3 Electrotactic Devices

Devices used for electrotactic studies all share commonalities and include 3 basic parts. These parts are the electrotactic chamber, the agar bridges, and electrodes. The resistance of the chamber can be calculated from the geometry in 3 dimensions and the voltage gradient is dependent solely on the cross-sectional area. Agar bridges serve to separate the electrotactic chamber and the cells within it from the products of hydrolysis at the electrodes. Typically, devices use Ag/AgCl ([96], [100], [127]), platinum ([26], [27]), or carbon ([128]) electrodes to generate the electric field. For two electrode setups involving agar bridges, it is not necessary to use inert electrodes if the agar bridges are sufficiently large.

The most conventional device is that outlined by Song et al. [63] and variations of it. This device is fabricated directly onto a Petri dish and is compatible with time lapse microscopy. Other devices are typically fabricated from PDMS and bonded to glass. The simplest microfluidic device is that used by Lin et al. which is fabricated by laser cutting a channel .5 mm wide x 1.5 cm long x .1 mm tall into Melinx plastic [26]. Two 200 µL pipette tips were aligned with each end of the channel and then attached with adhesives. Platinum wire electrodes were placed into the media reservoirs in the pipette tips and
experimentation lasts only 30 minutes to 2 hours to prevent poisoning of the cells via electrode products.

Other devices improve on the conventional geometry by altering the cross-sectional area of the device throughout the chamber. For instance, similar to the Lin device, a device constructed by Huang et al. is fabricated from PMMA sheets to provide inlets and outlets for media and agar bridges, while a piece of double sided tape is cut in the form of a microchannel [99]. The use of double sided tape as the microfluidic chamber is actually fairly common and is used in other electrotactic devices, often in conjunction with PMMA [129]. The width of this channel changes in three stepwise increments so that the electric field in each section of the channel is constant. This allows testing multiple electric fields at once. However, it adds in fluid flow as a factor because the media will be flowing more quickly in the section of the channel with the smallest width. Tsai’s device improves on this by having a device with multiple inlets as shown below in Figure 4 [96].
As shown, the widths of the side channels are varied so as to allow part of the electrical current through, creating three loops in parallel with each other. Controlling the widths of those channels alters the equivalent resistance of each respective loop, thereby controlling the current through that loop. By controlling the current through the channels of known resistance (sections I, II, and III) the voltage drop across each section of the electrotactic chamber is controlled.

Electrotactic chambers have also been incorporated into bioreactors in order to measure such cellular responses as nitrous oxide production [128]. Other devices incorporate on-chip electrodes fabricated out of indium tin oxide (ITO) ([130]) or metallic silver ([131]). Such devices allow unique geometries to be used and can bypass the use of agar bridges for low voltage applications. The wide range of devices that have been and can be fabricated for use in studying electrotaxis is promising for future work in electrical stimulation and detection of migrating cells.

Figure 4. Device diagram in which the electric field changes throughout the channel without altering fluid flow. Adapted from [96].
2.3.1 Microfluidic Devices

Recently, there has been a surge in the emerging field of microfluidics which utilizes easily manipulated polymers such as polydimethylsiloxane to create chambers with specific geometry on the micro and nano scale [132]–[134]. There are several manufacturing processes involved, most commonly photolithography and chemical etching, to create microfluidic devices of highly complex design [135]. The length scale of microfluidic devices make them especially applicable to cellular studies and as such, common types of microfluidics center around biochemical systems, including gradient generators [41], biochemical sensors [136], [137], single cell analysis [138], and cell sorting devices [139]. In all of these cases, microfluidics offer several advantages over bulk systems, i.e. Petri dishes or cover slips. Namely, flow within microfluidics is strictly laminar, which allows only diffusive chemical mixing rather than conventional. This also allows the shear stress at the surface of cell bodies to be calculated or modeled. The amount of volume used in such devices is much smaller than that of a bulk counterpart, making the use of expensive drugs and other assays more efficient. Because of the ease of reproduction through microfabrication, devices are consistent between experiments. At the length scale of microfluidic devices, electrokinetic phenomena become relevant in that the forces associated with them are of the same order of magnitude as shear and drag forces. For instance, dielectrophoresis has been utilized in the separation of live and dead yeast cells [140] and for the sorting of suspended MCF-10A and MCF-7 cells [141].

2.3.2 Unique Benefits of Electrotactic Devices

Electrotactic platforms are in general much simpler than their counterparts for other modes of directed motility and offer unique advantages. Chemotactic studies
require either the formation of a chemical gradient which remains constant over space and time within a microfluidic device or the addition of chemokines manually via pipette. Gradient generator devices use diffusion and mixing to establish a primary gradient which decreases in magnitude over the course of its length, allowing only a small window of applicable space where the gradient is relatively constant [41]. Moreover, as cells consume the chemical of interest (e.g. EGF), the gradient is further damaged for cells downstream. Additionally, as cells move up and down the chemical gradient, they are exposed to different absolute values of the chemokine which, if they target a signaling pathway which operates on a threshold, may induce a behavioral or phenotypic change. This is also true for platforms used for studying haptotaxis and durotaxis. Although each of these modes is fundamentally interesting and useful, voltage gradient platforms have much more versatility and are often more robust.

Other types of directed migration platforms involved micropatterned surfaces with protein or other ligand coats including fibronectin and collagen. Using microprinting, a two dimensional surface can be reduced to a single dimension, in the case of using printed lines, which has been shown to mimic the three dimensional space of the in vivo environment [142]. This is due to cells within the body utilizing fibers in the ECM for transportation which are better simulated by the one dimensional area on a printed line. Moreover, the surface can be custom tailored for specific assays in order to test polarity [51]. The use of these patterns to induce polarity has been studied extensively and has been shown to direct cell movement by using “teardrop” shaped patterns [143], [144]. Cells typically moved toward the wide end of the droplet, as they are capable of extending cell processes in that direction while retracting and dissolving
focal adhesions from the tapered end before jumping to the next tear drop in the design. This phenomenon has been utilized for one of very few substrate adherent cellular separation techniques, coined by the authors as “planar cell chromatography” [145]. This technique utilizes the difference in motility of Rho and Rac mutants and gives cells the ability to move around each other by allowing two jump locations from the wide end of each tear drop. Although this type of cellular separation could not be performed in a steady-state, continuous mode, it proves that cells can be separated and localized to specific areas without requiring them to be in suspension or otherwise disturb cell-ECM adhesion.

Electrotaxis offer significant advantages, especially when investigating responses on a small time scale. The electric field is established immediately upon application and does not require complex mixing systems to establish. It does require the use of agar salt bridges to separate the cellular environment from the electrode products which are generated for voltages over 1.23 volts. These bridges, however, are typically external from the device of interest. Because the electric field can be established on an instantaneous time scale, it offers the possibility of rapid switching, effectively swapping the source and sink in real time with the flick of a switch. To do an analogous experiment with chemotaxis, it would require a highly complex system of incorporated valves and pumps and probably involve pneumatic channels. Additionally, as cells move up and down the electric field, the absolute value of the voltage drop across the cell membrane does not change. A cell very close to the anode will experience the same field strength as one in the middle of the chamber. The field strength also does not decay over space or time when confined to a local geometry. These benefits make studying electrotaxis a
highly appealing technology for controlling cell migration as opposed to other guidance methods such as chemotaxis.

If voltages below the standard potential of water are applicable to a system, electrodes can be applied directly into the soluble environment without generating electrode products [146]. This has been achieved by using chemical etching on an indium titanium oxide (ITO) coated glass slide to create invisible electrodes which can be directly connected to an external power supply [32]. Because chemical etching relies on the same masking procedures used in device manufacture, spatial resolution on the same order of magnitude can be used to define electrodes with well-defined and complex geometries such as interdigititation [131].

2.3.3 Current Limitations

While most cell migration assays involving electrotaxis involve the use of a two dimensional substrate, there exists a need to develop more physiological platforms. Sun et al. has improved on the conventional electrotactic device by incorporating a three dimensional scaffold [30]. Ordered, three dimensional scaffolds are more representative of the in vivo environment [147]–[153]. Work has been done in our lab and others using pseudo one dimensional patterns as representative of the three dimensional environment [154]–[156]. However, that has not been applied to electrotactic systems outside of the work presented herein. Huang et al. has partially filled this gap by investigating electrotaxis of 3T3 mouse fibroblast cells through microfluidic channels [25]. Channels, although also pseudo one dimensional, differ greatly from fibers, especially with regards to electrical resistance and diffusion rates for soluble factors. Using a combination of microcontact printing and alternative electrode placement, our approach aims to close the
gap in current electrotactic devices by adding one dimensionality as well as electric field non-uniformity.

2.4 Summary

In short, electrotaxis is a very poorly understood type of directed cell migration. Although it shares certain pathways with chemotaxis, and may be influenced by chemical receptors known to affect chemotaxis [116], there is not complete overlap between the two migration types [24], [157]. E-cadherin is involved, at least in part, with electrotaxis of confluent cell layers [76]. Metastatic cancer cells typically display robust electrotaxis. E-cadherin, a known tumor suppressor [158], may also be involved there. Many studies concerning electrotaxis utilize platforms which may not capture important aspects of the physiological environment [63]. The work presented in this thesis looks to close the gap in understanding concerning the role of E-cadherin and cell-cell contacts in electrotaxis of both non-transformed and cancerous cells by using quantitative analysis and novel, physiologically relevant platforms.
3. Methods

This chapter includes detailed descriptions of the materials used in this thesis as well as the processes for preparation of devices and assays, as well as computational analysis of data. First, cell culture is discussed for all cell types, including those which are metastatic and those which are non-transformed. Fabrication steps for all devices used in this work, including the two dimensional platform, the invasion assays, and our new electrotactic device, are described. Image acquisition and the automation of image analysis are described both within this chapter and in Chapter 5, which focuses on the technical verification of the automated algorithm used. Finally, all quantification of important parameters is described in their respective equations within this chapter.
3.1 Cell culture

Cells were cultured by following these specific protocols. All cells were used within 20 to 25 passages of each other. Cells were never at passage numbers higher than 40. Special consideration was taken to prevent culture dishes remaining at confluence for prolonged periods of time.

3.1.1 MCF-10A

MCF-10A non-transformed human mammary epithelial cells were obtained from ATCC. 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 μg/mL insulin (Sigma), 0.5 μg/mL hydrocortisone (Sigma), 20 ng/mL EGF (Peprotech) and 0.1 μg/mL cholera toxin (Sigma) and maintained under humidified conditions at 37°C and 5% CO2. Cells were passaged every 3 days by dissociating fully confluent monolayers with 0.05% trypsin-EDTA (Invitrogen) and suspending cells in DMEM/F12 supplemented with 20% horse serum and 1% penicillin/streptomycin [159]. After two washes, cells were diluted 1:4 and plated in fresh growth medium. MCF-10A empty vector cells (PLKO) and MCF-10A E-cadherin knockdown cells (MCF-10A-E-cad) were a kind gift from Senthil Muthuswamy at Harvard Medical School. These MCF-10A derived cells were passaged in the same manner as parental MCF-10A cells.

3.1.2 MDA-MB-231

MDA-MB-231 human metastatic breast cancer cells were also obtained from ATCC. Cells were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM High Glucose, Invitrogen) supplemented with 10% fetal bovine serum
(Invitrogen) and 1% penicillin, streptomycin (Invitrogen). Cells were maintained in humidified conditions at 37°C and 5% CO2. Cells were passaged regularly by dissociating 80% confluent monolayers with 0.05% trypsin-EDTA (Invitrogen) and suspending cells in growth medium. Cells were diluted 1:8 and plated in growth medium. MDA-MB-231 cells expressing E-cadherin (231-Ecad) and a corresponding empty vector control cell line (231) were a kind gift from Senthil Muthuswamy at Harvard Medical School. These cells were passaged in the same manner as parental MDA-MB-231 cells.

3.1.3 Cell Labeling

CellTracker Red CMTPX Dye (ThermoFischer Scientific) was used to make cells visible under TexasRed fluorescence. Cells were dyed using 1 mL of dye media produced by the manufacturer’s recommendation. CellTracker (50 μg) was dissolved in DMSO (7.826 μL) in order to solubilize the dye. Of this, aliquots of 1 μL were added to 1 mL of growth media to produce dye media. In order to dye the cells, media was aspirated from the dish and replaced with 1 mL of dye media. Dishes were left to sit for 1 hour while the dye perfused into the cytoplasm of cells before being rinsed off and replaced with standard growth media. Cells were then used experimentally via the relevant protocol for that experiment.

3.2 Electrotactic chamber fabrication

The electrotactic chamber was assembled similarly to that described by Song [63] and further developed for our work [160]. Briefly, polystyrene dishes (60 mm) were taken and marked with lines 12 mm apart. Number 1 glass coverslips (22 x 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 x 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 x 22 mm gap. Dishes were then left to dry for at least 12 hours while being sterilized under ultraviolet light and were then stored for up to 4 weeks.

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

### 3.3 Electrotactic chamber preparation

Dishes were then coated with a 10 µg/mL solution of fibronectin (Invitrogen) in PBS (Invitrogen) for 1 hour prior to cell seeding. The epithelial cell solution (500 µL) was seeded into the PDMS well placed between the coverslips at varying concentrations (~10^3-10^5 cells/mL) and left to adhere for at least 12 hours 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 define a chamber with dimensions 12 mm x 22 mm x 0.15 mm. After closing the chamber, media was replenished once more before imaging.

### 3.4 Non-uniform electric field generator

The non-uniform electric field generator was conceived, modeled, designed, and fabricated by the following steps. Devices were originally produced using electron beam
evaporation to deposit gold layers onto glass. However, the following protocols utilize PDMS as a more readily available and workable substrate. Additionally, aluminum electrodes were substituted and found to work compatibly with the device and cells.

3.4.1 Electric field simulation

MATLAB’s partial differential equation tool was used to solve Gauss’s law in differential form (Equation 1):

\[
(\nabla \cdot E) = \frac{\rho}{\varepsilon}
\]  

(1)

where \((\nabla \cdot E)\) is the divergence of the electric field, \(\rho\) is the space charge density, and \(\varepsilon\) is the coefficient of dielectricity. The electrodes are specified to have Dirichlet boundary conditions with a voltage drop of 1 V from anode to cathode. The remaining surfaces are described as having no electric flux.

3.4.2 Non-uniform electric field generator device fabrication

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 partially cured at 80°C for 10 minutes in an unmodified plastic dish (100 mm diameter). Aluminum electrodes were cut using a computer generated stencil, sterilized, and pressed flat. After the PDMS mixture was partially cured to the point where convection currents in the solution were negligible, the electrodes were placed onto the surface, positioned according to a computer generated mask, and held in place via surface tension. The solution with the electrodes placed continued to cure at 80°C for over 1 hour. Devices were then cut to size to fit over a standard glass slide. Walls of 3140 silicon adhesive were used to define a cell culture region such that the leads of the electrodes extended out from under the silicon walls. The
devices were sterilized in UV light prior to use.

3.4.3 Non-uniform electric field generator device preparation

Devices were coated with a 10 $\mu$g/ml solution of fibronectin (Invitrogen) in PBS (Invitrogen) for 1 hour prior to cell seeding. A solution of epithelial cells (2 mL) was added to the PDMS well at a concentration of $\sim 10^3$ cells/ml, and cells were left to adhere for at least 2 hours in incubation. After rinsing with growth media to removed non-adherent cells, a glass slide roof was applied to the top of the silicon walls to encapsulate the cell growth media to inhibit contamination and prevent evaporation of media.

3.5 Microcontact printing

SU-8 2010 (Microchem) was spin-coated onto 3” silicon test wafers (Silicon Sense). The coating was exposed to UV light using a Quintel 4000 mask aligner through a chrome/soda lime mask (Front Range PhotoMask). Non-cross-linked portions were etched away using SU-8 developer (Microchem) leaving 3D negative features of lines of various widths. PDMS was mixed with cross-linker at a 10:1 ratio (Dow Corning) and was poured over the molds and cured for at least 2 hours at 80 C. The cured PDMS was extracted from the silicon wafer and stamps which displayed the patterned lines were cut from it. Stamps were cleaned and then coated in fibronectin by rocking overnight in a 1% solution of fibronectin in PBS. Patterns were transferred to the electrotactic device by pressing the stamp pattern-side-down for 30 seconds and then leaving it under its own weight for 1 hour. The devices were then coated with a 0.2% solution of Pluronic (Sigma-Aldrich) in PBS for 15 minutes in order to block cell adhesion to any areas not coated with fibronectin lines. Cells were then seeded onto the device and given at least 2 hours to adhere onto the lines before experimentation.
3.6 Transwell invasion assay

Transwell invasion assays were performed using an 8.0 micron pore size polystyrene cell culture insert (Corning). Matrigel (Corning) was diluted into a gelling solution by mixing 40 μL of Matrigel with 60 μL of cold serum free media. The gelling solution was kept on ice and deposited evenly onto the upper face of the cell culture insert. The insert was then placed into a 6-well plate and left for 1 hour at 37°C to gel. Media was added to both partitions of the insert so that 2 mL were above the insert and 3 mL were below. To the 2 mL in the upper partition, 2500 cells were added. Cells must be dyed prior to seeding in the invasion assay in order to ensure visibility by the fluorescent microscope. Cells were given 6 hours in the presence or the absence of an electric field driven across the membrane of the insert. After the 6 hours ended, the upper face of the inserts was wiped with a cotton-tipped swab. The bottom face of the inserts were then imaged under fluorescent light in order to visualize cells which passed through the pores within the membrane and cells were counted.

3.7 Image acquisition

Imaging was performed on an AxioVert 200 M inverted microscope (Zeiss). Devices were maintained at 37°C and 5% CO2 for 6 hours. Current was delivered to the chamber via two agar bridges (6” x 7 mm ID) which were bent under flame to fit beneath the condenser of the microscope. The agar bridges were comprised of 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 bridges was found to help stabilize 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 1M KCl solution. KCl reservoirs were kept at approximately equal liquid height as the media in the device to prevent syphoning of fluid. After 6 hours, no syphoning of KCl into device media was observed. Phase images were taken every 5 minutes.

### 3.8 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 with epithelial cells, a custom script was written that accurately identified and tracked cells with ~80% efficiency, defaulting to manual tracking when necessary (Fig. 1). Isolated cells were tracked with using the manual tracking interface reported previously [160]. Cells which divided during the middle of the video were discarded, as well as cells which died, detached from the substrate, or were otherwise compromised. In the case of isolated cells, colliding or attaching with another cell also led to discarding of the track. Cells which departed the field of vision were tracked until that time, and positional data up until the time of departure were compiled with the remainder of the data.

### 3.9 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 Equation 2,

\[
D = \cos(\theta)
\]  

(2)

where \( \theta \) 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 Equation 3,

\[ P = \frac{x}{l} \]  

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.

Mean square displacement (MSD) was calculated from Equation 4,

\[ MSD(\Delta t) = \sum_{c=1}^{n} \left( x(\Delta t, c) \right)^2 \]

where \( x(\Delta t, c) \) represents the displacement of a cell between its position at time, \( t \), and \( t + \Delta t \) at instance, \( c \). \( n \) is the number of possible instances for which the displacement at \( \Delta t \) can be calculated.

Because the linearity of the MSD plots is indicative of how ballistic migration is, the MSD curves were fitted to a power law curve using MATLAB’s nlinfit command,

\[ MSD(\Delta t) = \alpha (\Delta t)^\beta \]

where \( \alpha \) and \( \beta \) are calculated coefficients. The power law exponent, \( \beta \), reflects the degree to which cell migration is ballistic.

Additionally, speed and persistence where derived from the MSD by fitting to Equation 6 [161], [162]:

\[ MSD(\Delta t) = nS^2 \left[ P \Delta t - P^2 (1 - e^{-\Delta t/P}) \right] \]

where \( n \) is dimension number (here \( n = 2 \)), \( S \) is speed, and \( P \) is persistence in units of time. Persistence in this equation reflects the amount of time a cell spends traveling in the same direction in a persistence random walk model.
We have previously introduced the characteristic recovery 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 nlinfit:

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

(7)

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

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

(8)

Statistical analysis was done using the Statistics Toolbox in MATLAB.
4. Collective migration exhibits greater sensitivity but slower dynamics of alignment to applied electric fields

Published in Cellular and Molecular Bioengineering (2015)
Lalli, M.L., Asthagiri, A.R.
Cell Mol. Bioeng. 2015. 8:247-257
4.1 Abstract

During development and disease, cells migrate collectively in response to gradients in physical, chemical and electrical cues. Despite its physiological significance and potential therapeutic applications, electrotactic collective cell movement is relatively less well understood. Here, we analyze the combined effect of intercellular interactions and electric fields on the directional migration of non-transformed mammary epithelial cells, MCF-10A. Our data show that clustered cells exhibit greater sensitivity to applied electric fields but align more slowly than isolated cells. Clustered cells achieve half-maximal directedness with an electric field that is 50% weaker than that required by isolated cells; however, clustered cells take ~2-4 fold longer to align. This trade-off in greater sensitivity and slower dynamics correlates with the slower speed and intrinsic directedness of collective movement even in the absence of an electric field. Whereas isolated cells exhibit a persistent random walk, the trajectories of clustered cells are more ballistic as evidenced by the superlinear dependence of their mean square displacement on time. Thus, intrinsically-directed, slower clustered cells take longer to redirect and align with an electric field. These findings help to define the operating space and the engineering trade-offs for using electric fields to affect cell movement in biomedical applications.
4.2 Introduction

Extracellular electric fields are commonly found within the body in both healthy and diseased tissue. Transepithelial potentials (TEPs) on the order of tens of millivolts have been measured in tissues, such as skin, breast and prostate ducts [2], [17], [18]. Larger potentials on the order of hundreds of millivolts are generated by the flow of blood through the human circulatory system [19]. Rapid cell growth and associated significant alterations in surface charges induces an electric field between the tumor environment and regions of healthy tissue adjacent to it [21].

These extracellular electric fields play an important role in physiological processes. It is well known that reorientation and extension of neuron processes are influenced by electric fields [9]. Meanwhile, wounds in tissues, such as the skin, compromise the TEP and produce an ionic current, providing a stimulus for directed cell migration to close the wound [24]. For example, currents of up to 1 μA/mm of wound perimeter and electric fields of up to 2 V/cm were observed in wounds of skin epithelium [2]. In fact, imposing an opposing electric field is sufficient to overwhelm other stimuli and reverse wound closure, with cells migrating away from the wound edge [11], [24], [104]. The alignment of cell migration within externally applied electric fields, known as electrotaxis, has been reported in a number of cell systems, including fibroblasts, endothelial cells and normal and cancerous epithelial cells [26], [30], [59], [96], [99], [128].

For tissues such as the epithelium and endothelium, understanding electrotaxis particularly in the context of collective movement is important. Directed collective migration plays an important role in processes such as wound healing, angiogenesis and
the metastasis of cancer cells away from the primary tumor [66], [69], [163]–[165]. Geometric confinement, substrate stiffness and other microenvironmental parameters are known to affect the ability and efficiency of collective movement [73], [74], [166]. With electric fields already being applied in clinical applications, such as drug delivery, hyperthermic eradication of tumors, spinal cord regrowth, and ulcer healing [12], [167]–[169], it is essential to understand the effect of electric fields on cell migration in surrounding tissues where cell-cell interactions are prevalent. Furthermore, a quantitative understanding of the effect of electric field on the dynamics of collective migration would offer insights into utilizing this microenvironmental property to tune multicellular rearrangements in applications such as tissue engineering.

Collective movement is fundamentally different from the migration of isolated cells. Migration within a cluster requires the maintenance of intercellular adhesions and cell-cell signaling complexes. During collective migration, cell-cell adhesions allow for mechanotransduction and the propagation of correlated movement [74], [170]. Likewise, gap junctions within an epithelial layer mediate direct intercellular exchange of second messengers, particularly relevant to electric fields as they affect the distribution of calcium and other ions [171], [172]. Furthermore, the movement of clustered cells may be contact-inhibited and constrained by the lack of space to extend protrusions [173]. While both isolated and clustered cells remodel the underlying matrix and produce and consume growth factors [174], these processes are likely to be significantly different in the two situations due to differential ligand processing at the higher local density of cells in a cluster and due to the effect of cell-cell interactions on the distribution of forces that act on and help to remodel the matrix.
Given the complexity of collective movement, it is unclear how an electric field will affect collective migration and how this effect will differ from the response of isolated cells. In this study, we examine this question by conducting a quantitative comparative analysis of the electrotaxis of isolated and clustered cells using the MCF-10A non-transformed human mammary epithelial cell line as a model system.
4.3 Results

4.3.1 Clustered MCF-10A cells are more sensitive to electric field than isolated counterparts

To investigate collective movement in an electric field, the trajectory of isolated and clustered MCF-10A cells were observed by time-lapse microscopy in the absence of an electric field or in the presence of different electric field strengths. In the presence of an electric field of 1.0 V/cm, both isolated and clustered cells were observed to migrate toward the anode (Fig. 5A). Meanwhile, in the absence of an electric field, there was no apparent directional bias in the movement of the cells. Visualizing the trajectory of twenty randomly selected cells qualitatively confirmed the electrotactic movement of both isolated and clustered MCF-10A cells (Fig. 5B). Interestingly, from this small sample of trajectories, isolated cells appeared to require an electric field strength of 0.51 V/cm to direct their migration toward the anode; in contrast, clustered cells seemed to exhibit directed migration within a weaker electric field of 0.26 V/cm.

To investigate more quantitatively whether isolated and clustered cells have different sensitivity to electric field strength, we quantified the trajectories of approximately 2,500 isolated and 7,000 clustered cells across five levels of electric field. We determined the percentage of cells migrating toward the anode of the electric field: cells whose final position was closer to the anode than their initial position were scored as having migrated toward the anode (Table 1). Cluster size ranged from dozens of cells to hundreds of cells reaching partial confluence. Even at the lowest applied electric field of 0.13 V/cm, a much larger fraction of clustered cells moved toward the anode in comparison to isolated cells. Meanwhile, under control conditions in the absence of an
Figure 5. (A) Snapshots taken at times 0, 75, 150, 225, and 300 minutes with five isolated (top row) and clustered (bottom row) cells labeled. The white-dashed grid is centered at the same position in each image to provide a constant positional reference. Scale bar = 100 µm. The anode is in the positive y-direction. (B) Examples of 20 trajectories of isolated (top row) and clustered (bottom row) cells moving in the absence (left column) and presence of electric fields of 0.13, 0.26, 0.51, and 1.0 V/cm. A solid black circle indicates the origin (0,0) and is the starting point for every trajectory. In all cases, the anode is in the positive y-direction, and the x-direction is orthogonal to the electric field.
electric field, both isolated and clustered cells showed no appreciable bias toward the anode.

### Table 1. Percentage of cells migrating toward the anode of the electric field.

<table>
<thead>
<tr>
<th>Electric field</th>
<th>% Cells aligned</th>
<th>Sample size$^\S$</th>
<th>% Cells aligned</th>
<th>Sample size$^\S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 V/cm</td>
<td>51.8</td>
<td>456</td>
<td>51.9</td>
<td>1708</td>
</tr>
<tr>
<td>0.13 V/cm</td>
<td>57.0</td>
<td>377</td>
<td>70.1</td>
<td>1491</td>
</tr>
<tr>
<td>0.26 V/cm</td>
<td>73.8</td>
<td>477</td>
<td>84.2</td>
<td>1103</td>
</tr>
<tr>
<td>0.51 V/cm</td>
<td>83.9</td>
<td>615</td>
<td>95.0</td>
<td>1550</td>
</tr>
<tr>
<td>1.0 V/cm</td>
<td>89.5</td>
<td>535</td>
<td>99.0</td>
<td>1240</td>
</tr>
</tbody>
</table>

* Cells with net displacement in the direction of the anode were scored as aligned with the field.

$^\S$ Sample size indicates the number of cells observed over 3 independent trials.

To gauge the extent to which cell movement is aligned with the electric field, we quantified the directedness as the cosine of the angle between the cell displacement vector and the axis of the electric field (Fig. 6). A directedness value of 1 or -1 would be indicative of a net directional bias toward the anode or the cathode, respectively.

Our measurements show that for a particular magnitude of electric field, clustered cells become more aligned than isolated cells within the 6 h observation window (Fig. 7). In addition, we observe a change in the threshold required to initiate an electrotactic response. The lowest applied electric field (0.13 V/cm) is sufficient to increase the directedness of clustered cells above the baseline control case where no electric field is presented as indicated by a two-tailed t-test comparing the distributions of directedness in each case ($p < 0.01$). In contrast, isolated cells require an electric field of 0.26 V/cm or
greater in order to shift from their baseline response (p < 0.01). A weaker electric field of 0.13 V/cm is insufficient to induce directedness of isolated cells (p > 0.01).

**Figure 6.** A schematic of the trajectory of a cell migrating within an electric field. The black line indicates the electric field vector from cathode to anode and the blue line shows the trajectory of the cell in time. The red line represents the overall displacement of the cell. The angle between the electric field vector and the cell displacement vector is labeled $\theta$.

To examine the directedness of cells at a single-cell level, we constructed circular histograms (rose plots) of the angle between the vector of displacement after the observation window and the axis of the electric field (Fig. 8). Using the omnibus test in the Circular Statistics Toolbox for MATLAB [175], we tested the null hypothesis that the distribution of angles is uniform. Among isolated cells exposed to an electric field, the
null hypothesis was rejected for electric fields at or above 0.26 V/cm (p < 0.01) but not at 0.13 V/cm, further confirming that a potential gradient greater than 0.13 V/cm is needed to induce electrotaxis of isolated cells. In contrast, the rose plot for clustered cells scored as non-uniform at all applied electric fields. The v-test confirmed that the directional bias is indeed toward zero degrees, the direction of the anode, in all cases for which the omnibus test rejected the null hypothesis. These results show that clustered MCF-10A cells are indeed more sensitive to an electric field than isolated cells, consistent with similar enhanced sensitivity of clustered MDCK I and MDCK II cells reported previously [76].

**Figure 7.** Directedness as a function of electric field. The directedness was calculated as the cosine of the angle of the final displacement vector of a cell with respect to the cathode-anode axis. The mean directedness for isolated (red) and clustered (blue) cells is depicted for electric fields of 0, 0.13, 0.26, 0.51, and 1.0 V/cm. Error bars indicate standard error of the mean.
Interestingly, the rose plots for clustered cells exhibited an unexpected feature in the control case where no external electric field is applied. The omnibus test revealed that the displacement direction of clustered cells was non-uniform. In contrast, isolated cells exhibited a uniform distribution of displacement angles under control conditions. Further analysis with the v-test revealed that the direction of movement of clustered cells was non-uniform with a range of angles showing bias, which included 45° and 135°. These results are attributed to the fact that clustered cells initially share an orientation of movement with their neighbors which is stochastic in nature. These subgroups effectively

**Figure 8.** Rose plots of the angle of the displacement of isolated cells and clustered cells migrating within electric fields. (A) Rose plots of isolated cells in the absence of an electric fields (left column) or the presence of an electric field of 0.13, 0.26, 0.51, or 1.0 V/cm. The anode and cathode are at 0 and 180 degrees, respectively. In the absence of an electric field and at an electric field of 0.13 V/cm, the p-value of the omnibus test was greater than 0.01. At all electric field strengths greater than 0.13 V/cm, the p-value of the omnibus test was less than 0.01. These distributions also had p-values less than 0.01 for the v-test with selected mean of 0°. (B) Rose plots of clustered cells in the absence of an electric fields (left column) or the presence of an electric field of 0.13, 0.26, 0.51, or 1.0 V/cm. Again, the anode and cathode are at 0 and 180 degrees, respectively. At all electric field strengths greater than 0 V/cm, the p-value of the omnibus test was less than 0.01. These also had p-values less than 0.01 for the v-test with selected mean of 0°. At 0 V/cm, there was a significant difference between the observed distribution and a uniform distribution although when analyzed with the v-test, the null hypothesis could be rejected for a range of angles which included 15°, 45°, 90°, and 135°.
reduce the sample size of independent angles, thus giving rise to a multi-modal distribution of displacement angles across the population.

4.3.2 Cell clustering and an external electric field additively enhance ballistic and persistent cell movement

Our analysis shows that isolated cells have no directional bias in the absence of an external field and acquire directed movement toward the anode upon exposure to a suprathreshold electric field. These results suggest the hypothesis that isolated cells move in a persistent random walk in the absence of a field and acquire a more ballistic trajectory toward the anode upon exposure to an electric field. In contrast, our analysis demonstrates that even in the absence of an external field, subgroups of clustered cells move in a directed manner, and the external electric field acts to reorient these subgroups in the direction of the anode. Therefore, we hypothesized that clustered cells may exhibit ballistic movement even in the absence of an electric field.

Since cells with a more ballistic migration trajectory would be expected to show greater persistence, we tested this hypothesis by analyzing the persistence of clustered versus isolated cells in the absence and presence of an electric field. Persistence was quantified as the ratio of net displacement to total distance traveled throughout the observation window (Eq. 2). We found that the persistence of clustered cells was nearly two-fold greater than that of isolated cells in the absence of an applied electric field. The effect of applying an electric field on persistence was modest but statistically significant via ANOVA (p < 0.01). Exposure to an electric field of 0.51 V/cm and greater increased the persistence of both isolated and clustered cells (Fig. 9).
Notably, the difference in the persistence of isolated cells and that of clustered cells remained approximately constant at all electric fields, suggesting that the effects of cell clustering and the electric field on the directed migration of MCF-10A cells are additive. To examine this possibility more quantitatively, we calculated the persistence of clustered cells that would be predicted if the effect of electric field and clustering were

**Figure 9.** Persistence of cells under varying electric field strengths. The persistence was calculated as the ratio between net displacement and total distance traveled. The measured mean persistence (solid lines) for isolated (red) and clustered (blue) cells is shown for electric fields of 0, 0.13, 0.26, 0.51, and 1.0 V/cm. The predicted persistence for clustered cells (dotted blue) is calculated as the additive effect of the electric field and cell clustering (see Methods). Error bars for the measured persistence indicate standard error of the mean, and the error bars for the predicted persistence is computed by the propagation of errors of the measured values of persistence.
additive. The additive model predicts the persistence of clustered cells with ~90% accuracy (Fig. 9).

To further characterize the ballistic versus diffusive movement of isolated and clustered cells, we analyzed the mean square displacement (MSD) calculated from cell migration trajectories (Fig. 10 A and B). A ballistic particle exhibits a second-order dependence of MSD on the duration of observation while the MSD will increase linearly with the time of observation for a particle undergoing Brownian motion or a random walk model of cell migration [176]. For isolated cells, we observe that the MSD increases linearly with the duration of observation in the absence of a field and for low electric fields. However, at higher field strengths (e.g., 1.0 V/cm), the dependence of MSD on duration of observation begins to exhibit some upward curvature. In contrast, for clustered cells, the curves showing the dependence of MSD on the time of observation are clearly concave upward in the absence of the electric field and for all applied electric fields. Interestingly, we see a decrease in the MSD for clustered cells (Fig. 10B) at electric fields of 0.13 and 0.26 V/cm, which corresponds to an observed 20% decrease in the average speed of the cells (Fig. 10C). The speed is then recovered at higher electric fields, reflected in the increase of MSD for electric fields of 0.51 and 1.0 V/cm. Notably, the speed of isolated cells is approximately twice the speed of clustered cells for all values of the electric field.
In order to characterize the extent to which the migration path was diffusive versus ballistic, independent of cell speed, the dependence of MSD on the time of observation was fitted to a power law curve (Eq. 4) and the exponent ($\beta$) of the power law was determined (Fig. 10D). A $\beta$ value of 1 is consistent with a persistent random walk.

**Figure 10.** Mean square displacement of cells under varying electric field strengths. Mean square displacement (MSD) was calculated for (A) isolated cells and (B) clustered cells with time duration ranging from 0 to 6 h at 5 min intervals in the absence (red) or presence of electric field of 0.13 (blue), 0.26 (black), 0.51 (green), and 1.0 (magenta) V/cm. (C) Average speed of isolated (red) and clustered (blue) cells under varying electric field strengths. Cell speed is calculated as the path length of the cell divided by time. Error bars indicate standard error of the mean. (D) The MSD dependence on duration was fit to a power law and the corresponding exponent of the power law fit is shown for isolated (red) and clustered (cells) at electric fields of 0, 0.13, 0.26, 0.51, and 1.0 V/cm. Error bars represent 95% confidence intervals based on the power law fit.
while a $\beta$ value of 2 indicates purely ballistic motion. The $\beta$ value for clustered cells was 1.6 in the absence of an electric field. In contrast, the value of the exponent for isolated cells in the absence of an electric field was 1.03. These results demonstrate that in the absence of an external field, cell clustering provides supradiffusive and more ballistic character to cell movement.

The value of $\beta$ increased with electric field strength, indicating that cells move in a less random path when biased by an electric field. Notably, the difference in the value of $\beta$ between isolated cells and cell clusters remained fairly constant across all electric field strengths, thereby disentangling the effect of cell clustering and electric field on promoting ballistic cell movement. These results provide further evidence that the effects of cell clustering and the application of an electric field act additively, even if not entirely independently, on cell migration.

4.3.3 Clustered cells require longer time to reorient in an electric field

Since clustered cells are slower and significantly more ballistic and persistent in their motion, even when no electric field is applied, we reasoned that cells in clusters may take longer to orient themselves within an electric field than isolated cells. To examine this hypothesis, we identified cells that were initially moving toward the cathode and quantified their reorientation toward the anode. Cells whose displacement was toward the cathode during the first hour were filtered, and their mean directedness was determined over time.
As shown in Fig. 11A, the directedness of filtered cells initially trends more negative as we are considering only cells that move toward the cathode. The directedness then recovers as these cells achieve their steady-state orientations. In the control case without an electric field, the directedness approaches a steady-state value near zero, as would be expected for all cells in the long term without any external bias. In the presence of an electric field, cells recover to a directedness value greater than that observed under control conditions. At higher electric fields, the directedness reaches a higher steady-state value. At extremely high potential (1 V/cm), an order of magnitude greater than the threshold needed to initiate electrotaxis (approx. 0.13 V/cm), both clustered and isolated

![Graph A](image1)

![Graph B](image2)

**Figure 11.** Dynamics of acquisition of electrotactic directedness by cells initially aligned opposite to the cathode-anode vector. (A) Directedness over time of isolated (solid lines) and clustered (dotted lines) cells which were initially moving toward the cathode and remained on such a trajectory for the duration of the first hour of observation is shown in the absence (red) or presence of electric field of 0.13 (blue), 0.26 (black), 0.51 (green), and 1.0 (magenta) V/cm (B) The characteristic time of recovery was calculated by fitting to an exponential recovery model and solving for the time required to reach half maximal directedness (see Methods). The characteristic time of recovery for isolated (red) and clustered (blue) cells is shown for electric fields of 0, 0.13, 0.26, 0.51, and 1.0 V/cm. Error bars represent 95% confidence intervals based on the exponential recovery model.
cells achieve directedness in approximately the same timescale. This convergence suggests that cell-cell interactions may begin to break down at stronger electric fields. Except for the highest electric field, the directedness of isolated cells reaches a steady-state more quickly than that of clustered cells, consistent with our hypothesis.

To quantify the kinetics, the time course of the recovery in directedness was fit to an exponential function (Eq. 6) by taking the nadir of the curve as the initial point and the characteristic time needed to reach half-maximal directedness was determined (Fig. 11B). The timescale for the reorientation of clustered cells was approximately 2-8 times longer than isolated cells in the absence of an electric field and for electric fields weaker than 1.0 V/cm. Since the difference in speed between isolated and clustered cells is only two-fold at all electric fields (Fig. 10C), the slower movement only partially explains the retarded reorientation kinetics of clustered cells. These results support our hypothesis that the inherent ballistic and persistent movement of clustered cells is a significant contributing factor in the slower orientation dynamics of clustered cells in electric fields of low to moderate magnitude.
4.4 Discussion

In this study, we identify three significant features that distinguish the electrotaxis of clustered mammary epithelial cells from that of isolated cells. First, clustered cells are more sensitive to the magnitude of the electric potential than isolated cells: clustered cells achieve greater directedness at lower electric field strength. Second, cell clustering and an electric field have an approximately additive effect on the persistence of cell movement. Even in the absence of an external electric field, the migration trajectory of clustered cells is inherently more persistent than the random walk exhibited by isolated cells. The application of an electric field enhances this persistence but to a similar extent in both clustered and isolated cells, revealing that cell clustering and an electric field have separate, superimposable effects on the persistence of cell movement. Finally, we show that the inherent persistence of clustered cells renders them slower to align with an external electric field when compared to isolated cells. Thus, although clustered cells ultimately achieve a greater directedness at lower electric potential, the dynamics of their alignment are retarded by the extra time it takes to reorient cells that are already directed.

Clustered mammary epithelial cells align with an external electric field at a lower field strength than isolated cells. The effective potential (EP50) to reach half maximal fraction of aligned cells for clustered cells is approximately 0.2 V/cm whereas the EP50 for isolated cells is approximately 0.3 V/cm (Table 1). The higher electrotactic sensitivity of clustered MCF-10A cells is consistent with observations that MDCK I and MDCK II cells are more sensitive to an electric field when clustered [76]. In comparison to MDCK cells, MCF-10A cells form weak tight junctions due to reduced expression of ZO-1 [177], leading us to conclude that strong tight junctions are not necessary for the enhanced
electrotactic responsiveness of clustered cells. Meanwhile, E-cadherin-mediated adherens junctions seem to play an important role since the knockdown of E-cadherin abrogated the enhanced directedness of clustered MDCK cells in an electric field [76].

Our data suggest that cell clusters align to an electric field with greater sensitivity than isolated cells because cells in clusters are inherently more directed. We find that even in the absence of an external field, the migration trajectory of clustered cells is highly persistent (Fig. 9) and comparatively ballistic (Fig. 10). In contrast, the migration paths of isolated cells exhibit diffusive behavior characterized by a linear dependence of mean square displacement (MSD) with time. Furthermore, exposure to an external electric field increases the persistence of both clustered and isolated cells in an equivalent manner. Since the electric field does not have a comparatively stronger effect on the persistence of clustered versus isolated cells, we propose a model wherein the intrinsic persistence endowed by clustering underpins the enhanced sensitivity of clustered cells to an electric field.

At least two factors may contribute to the greater inherent persistence of cells in clusters. In the crowded environment of a cell cluster, a moving cell will collide with neighbors, thereby providing a non-stationary physical obstruction that constrains the direction in which neighbors can move. In addition to this physical obstruction, cell-cell adhesions provide the means for momentum transfer from one viscoelastic cell to another. Together, these mechanisms propagate the movement of one cell to bias neighboring cells, leading to collective directed movement greater than that observed in isolated cells. In the context of an electric field, a consequence of this inherent directedness is that aligning an individual cell in a cluster can have broader effects on its
neighbors, thereby making the clustered population more responsive to an electric field than isolated cells. We are pursuing experimental and mathematical modeling approaches to examine these mechanisms in greater detail. Meanwhile, in this study, we focus on delineating further the implications of the intrinsic directedness of clustered cells on their electrotactic response.

Although the intrinsic directedness of clustered cells enables them to achieve greater directedness at lower electric potential, the kinetics to achieve alignment is 2-8 fold slower in clustered cells (Fig. 11). We examined cells that were moving away from the anode during the first hour and quantified how long it took them to redirect toward the anode. Clustered cells are 2-4 fold slower than isolated cells to achieve directedness to the anode. In addition, in the absence of an electric field, clustered cells are 8-fold slower to recover to a random distribution of orientations, demonstrating that the slower orientation kinetics is a property associated with the clustered state. While clustered cells move two-fold slower than isolated cells (Fig. 10C), this disparity in migration speed only partially explains the 2-8 fold slower orientation dynamics of clustered cells. This analysis suggests that the inherent directedness of clustered cells is an additional significant factor in retarding the orientation dynamics.

Our findings have implications for our understanding of directed collective migration and for developing strategies to tune this physiologically significant mode of migration. Directed collective migration is a critical process in development and the progression of diseases, such as cancer [69]. In these contexts, external molecular fields, such as chemotactic or haptotactic gradients, play a prominent role in conferring directional bias [178]–[180]. It will be interesting to determine whether our finding that
collective movement enhances sensitivity but retards the kinetics of alignment to an electric field extends to other external fields that bias cell movement. Meanwhile, the results from this study reveal that cell clustering leads to a trade-off in sensitivity versus dynamics of electrotaxis. Our results provide deeper quantitative insights into this trade-off and help to define the operating space for technologies that seek to affect cell movement using electric fields in applications such as tissue engineering. In addition, electric fields are employed for reasons other than manipulating cell migration in a wide range of therapeutic applications, including drug delivery, hyperthermic eradication of tumors, spinal cord regrowth, and ulcer healing [12], [167]–[169]. In such situations, our findings offer insights into the potential off-target effects of the external electric field on the migratory behavior of cells in the exposed region and the possible consequences for cellular organization of the tissue.
5. Label Free Automated Tracking for Exploration of Effects of E-cadherin Expression on Electrotaxis

Under review in Cellular and Molecular Bioengineering (2016)
Lalli, M.L., Wojeski, B., Asthagiri, A.R.
5.1 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 electrotactic potential, with empty vector MCF-10A cells showing 16% higher directedness than cells with E-cadherin knockdown. Decreased electrotaxis is also observed in isolated cells at intermediate electric fields, suggesting an adhesion-independent role of E-cadherin in regulating electrotaxis. Additionally supporting 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.
5.2 Introduction

Collective migration is required in many physiological processes including tissue development and repair [65], [181]. 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 [69], [182], [183]. 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 [11], [166], [91]. 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 [76]. 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 resonance [184] or fluorescence [185]. For example, open access platforms such as ImageJ and Fiji provide tracking algorithms such as TrackMate [186] and the MOSAIC Suite [187], but both are designed optimally for fluorescently-labeled cells or cell parts. Fluorescent labeling aids automated tracking by localizing fluorescent signal to the nucleus [188] 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 Hoechst [189] or a histone 2B-GFP fusion protein such as H2BGFP [190].

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 [191]. 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 membranes [192] and has been used for tracking lineage of
cells within the zebrafish blastocyst, where fluorescent staining would be too difficult [193]. Ptychography, which enhances contrast by comparing diffraction patterns to brightfield images, has recently garnered attention as a label-free imaging technique [194]. 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.

Here, we develop a label-free tracking algorithm capable of identifying individual cells within a migrating cell cluster. We apply this algorithm to study the electrotaxis of clustered epithelial cells in a high throughput manner. We and others have previously shown showed that clustered cells exhibit better electrotactic response than isolated counterparts [76], [160]. We sought to investigate the role of the expression of E-cadherin, a cell surface receptor that mediates cell-cell adhesion [87], [89], in the enhanced electrotaxis of clustered cells. E-cadherin expression is often downregulated in cancer progression [195], [196] and epithelial derived cancer cells typically display robust electrotaxis [11], [76], [97]. Moreover, inhibition of E-cadherin with DECMA-1 or by Ca$^{2+}$ depletion eliminated electrotaxis in clusters of MDCK cells [76]. 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 60% 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.
5.3 Results

5.3.1 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 migration 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) (Fig. S1). 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. The algorithm then focuses on one cell, determining its movement trajectory longitudinally over time to the end of the experiment, and then returns to the initial frame to process the next cell.

A step-by-step walk through of how the algorithm segments and identifies the position of a cell is provided in Figure 12. First, the portion of the frame surrounding the initial coordinates of the cell is cropped for analysis (Fig. 12A). 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. 12B). 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 are stored separately (Fig. 12C). Then, a binary mask is constructed from the contrast enhanced image based on intensity (Fig.
12D). This binary mask is combined with the contrast enhanced image to remove regions initially determined to be cell-cell boundaries (Fig. 12E). After merging with the mask, a short erosion step is added to further separate regions of interest (Fig. 12F). This image is then merged with the highlight of intracellular structures, the result of which is increased pixel intensity toward the interior of the cell (Fig. 12G). A second thresholding is performed on this compound image in order to remove more space between cells (Fig. 12H). 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, another erosion followed by a size limited filter to remove any remaining background or cell fragments from being analyzed is performed (Fig. 12I). The coordinates of the cell center are determined as the centroid of the detected region of interest (Fig. 12J). 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.
Figure 12. Algorithm workflow for image processing. A. Original phase microscopy image of cell cluster focused on a relatively small area. B. Logarithmic contrast-enhanced image. C. Feature edge detection of contrast enhanced image via estimation of the intensity derivative. 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 of masked image. G. Combination of edge detection with opened image. H. Opening of combined image. I. Size filtering to reduce detected fragments and background noise. J. Identified cell center based on distance limitation is labeled in white. Scale bars on (shown on A and J) are 50 microns.
The segmentation process outlined in Figure 12 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 μm/min at any given point, which is a speed generally unattainable by a clustered epithelial cell. Additionally, the final determined ROI must by at least 21 μm². 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 ~80% efficiency, relying on human input ~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.

5.3.2 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 [156]. 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 1,716 cells were tracked in every instance and therefore retained identical initial coordinates.

**Figure 13.** 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.

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. 13).

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 minutes 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 4-8 μm, 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 μm.

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 ~4-8 μm. These results demonstrate that the automated algorithm performs within the variability of manual tracking.

5.3.3 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 toward 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. 14). 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 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.

**5.3.4 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 hours 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 is 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 Equations 7 and 8 (see Methods).

Cells within epithelial cell clusters took longer to align to the electric field than isolated cells (Fig. 15A). Among clustered cells, 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 4-fold increase in the magnitude of applied electric field in order to reorient 2.6-fold more quickly. Comparatively, 10A-shEcad cells reoriented 2.4-fold faster after only a 2-fold increase in the magnitude of the applied
electric field. In addition to increasing the sensitivity of reorientation kinetics to changes in electric field strength, reducing E-cadherin expression affected the time to orient at each fixed magnitude of applied electric field (Fig. 15B). Decreasing E-cadherin expression retarded reorientation at low and high electric field strengths, while speeding up reorientation at the intermediate electric field.

Figure 15. 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) Absolute orientation time of cells. Large characteristic orientation times are indicative of slow reorientation to an electric field. B) Fold change of orientation time between 10A-pLKO cells and 10A-shEcad 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.

For epithelial cells in isolation, both 10A-pLKO and 10A-shEcad cells reoriented in an electric field within ~1h, faster than when clustered (Fig. 5A). 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. 15B). 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.

**5.3.6 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 (Table 2).

Persistence of migrating cells was quantified as the net displacement over the observation time divided by the total path length taken by the cell [74], [197]. Only cells within epithelial cell clusters display a dependence of persistence on electric field strength (Fig. 16). 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 this differences in persistence are quantitatively modest, the large sample size made feasible by automated tracking confers statistical significance.
Table 2. Directedness of migrating cells and cell speed. Cell speed is listed as parenthetical values and reported in μm/min. Sample sizes are listed in brackets.

<table>
<thead>
<tr>
<th>Electric field</th>
<th>Clustered Cells</th>
<th>Isolated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLKO</td>
<td>MCF-10A⁻E-cad</td>
</tr>
<tr>
<td>0 V/cm</td>
<td>0.00</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>(0.44) (0.42)</td>
<td>(1.02) (0.76)</td>
</tr>
<tr>
<td>0.13 V/cm</td>
<td>0.35</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>(0.44) (0.45)</td>
<td>(0.90) (0.69)</td>
</tr>
<tr>
<td>0.26 V/cm</td>
<td>0.56</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>(0.41) (0.46)</td>
<td>(0.64) (0.91)</td>
</tr>
<tr>
<td>0.51 V/cm</td>
<td>0.83</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>(0.56) (0.52)</td>
<td>(0.66) (0.76)</td>
</tr>
</tbody>
</table>

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. 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 electrotaxis.
5.3.7 Calculation of migration properties is not dependent on tracking method

The above analysis examines migration properties based on trajectories acquired with the automated algorithm. We established that these trajectories are in close agreement with those identified manually. Therefore, 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 1,716 migration trajectories that were manually identified, twice independently.

**Figure 16.** Persistence of cells in various applied electric field strengths. Persistence is calculated for clustered epithelial cells (dotted lines) and isolated epithelial cells (solid lines) while electric fields of 0, 0.13, 0.26, and 0.51 V/cm are applied. Squares indicate MCF-10A$^{E-cad}$ cells while circles indicate PLKO cells. Error bars indicate the standard error of the mean.
Directedness was calculated for these cells and compared between methods (Fig. 17A, D). The directedness of cells was similar for both repetitions of manual tracking (Fig. 17A). 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. 17D). 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.

**Figure 17.** 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.
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. 17B); the same consistency was observed in calculating migration speed (Fig. 17C). Meanwhile, trajectories identified by the algorithm yielded values of persistence that were 15.2% lower than those calculated from manually-acquired trajectories (Fig. 17E). Speed also exhibits a systematic difference between manual and automated methods: speed calculated from tracks acquired by the algorithm was on average 15.7% greater than those estimated from manually-acquired trajectories (Fig. 17F). This systematic difference is a result of statistical noise in the automated tracking

**Figure 18.** Comparison of migration metrics as calculated through analysis of mean square displacement (MSD). Speed, persistence, and mean square displacement power law exponent were derived from fitting to Equations 4 and 5. First, both iterations of manual tracking were compared for the power law exponent of MSD (A), speed (B), and persistence (C). A linear fit intercepting the origin was determined (circles). The ideal fit (solid line) is a 1:1 direct proportionality. Second, the data from the automatically tracked cells was compared against the mean of the two manual iterations for the power law exponent of MSD (D), speed (E), and persistence (F) and a linear fit intercepting the origin was determined (circles).
method.

The exponent of the MSD power law was evaluated and the results of manual and automated tracking were compared (Fig. 18 A, D). It can be seen that the two methods have only small deviations from each other, with automated tracking resulting in 2.3% higher values of power law exponent. Additionally, power law exponent values were calculated as 1.6 - 1.8 over this range of electric fields using automated tracking of clustered cells (Fig. 19). These values are consistent with what has been reported previously in manually tracked clustered breast epithelial cells [160]. Equation 6 was

![Figure 19](image-url)

**Figure 19.** Power law exponent of mean square displacement of cells under varying electric field strengths. Electric fields of 0, 0.13, 0.26, and 0.51 V/cm were applied. Dotted lines indicate clustered MCF-10A cells and solid lines indicate isolated MCF-10A cells. Squares indicate MCF-10A-E-cad cells while circles indicate PLKO cells. Error bars indicate 95% confidence intervals.
used to calculate the MSD-derived speed (Fig. 18 B, E) and persistence (Fig. 18 C, F) terms. Speed calculated in this way was found to be underestimated by 6.3% and persistence was overestimated by 15.0%. Persistence in this calculation is the amount of time a cell spends travelling in a given direction before changing directions.
5.4 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 1,716 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-adjoining cells, individual cells must be identified (the segmentation problem) [198], [199]. 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) [200]. 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 5-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 40% 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. 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. However, based on the disproportionate 20% reduction in electrotaxis in response to a 40% downregulation in E-cadherin expression, we leave 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 [177].

In contrast to our findings in mammary epithelial cells, research in MDCK cells implicate E-cadherin as the chief regulator of collective electrotaxis [76]. Inhibition of E-cadherin by blocking with DECMA-1 or depleting extracellular CA\textsuperscript{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 [76]. First, to what extent blocking with DECMA-1 or depleting extracellular CA\textsuperscript{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 [201]. We have shown previously that EGF induces β-catenin signaling in MCF-10A cells seeded at a sub-confluent density [202]. E-cadherin also associates with and modulates the functionality
of other cell surface receptors, including the EGF receptor [203], [204], which has been implicated in regulating electrotaxis [8], [17], [97].

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 [195], [196], 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 [102].
6. Application of Electric Fields Promotes Invasion of Metastatic Cells through Crowded Environment

In preparation for submission
Lalli, M.L., Orzechowski, H., Astagiri, A.R.
6.1 Abstract

Cell migration in response to physical, chemical, and electrical stimuli is fundamental to the progression of metastatic disease. While many cell types are known to exhibit electrotaxis in vitro in mono-culture systems, it is unclear how invasive cells respond to electric fields while interacting with surrounding, non-transformed cells. Herein, we analyze the effect of E-cadherin expression on electrically-stimulated migration of MDA-MB-231 breast adenocarcinoma cells within a highly confined microenvironment. This microenvironment is generated by directly seeding MDA-MB-231 cells into a confluent layer of breast epithelial cells. To test the role that E-cadherin plays in the electrotactic response of invasive cells, an E-cadherin expressing line (231-Ecad) was acquired compared to the control cell line (231) which lacked E-cadherin expression. Our data show that 231-Ecad cells are 37% less directed with respect to an applied electric field while migrating through the confined environment of an immobile epithelial sheet compared to 231 cells. E-cadherin expression also plays a role in the dynamics of electrotactic response. E-cadherin expression results in a 28% increase in the time taken for invasive cells to reorient to an electric field. Moreover, we showed that despite being highly confined by a confluent layer of epithelial cells, MDA-MB-231 cells still showed non-Brownian, persistent migration. Using a standard transwell invasion assay, 231-Ecad cells are observed to be less able to penetrate a layer of Matrigel than control 231 cells. However, application of a weak electric field is sufficient to induce invasion at an equivalent level as control 231 cells. These findings help to elucidate the role of E-cadherin expression and the confinement of the microenvironment during electrotaxis in a heterogeneous cell culture of cancerous and non-transformed cells.
6.2 Introduction

Endogenous electric fields are prevalent in many physiological phenomena including development, wound repair, and progression of metastatic disease. Electric potentials at the millivolt level form across the epithelium, known as the transepithelial potential (TEP), within the skin, breast, and prostate [2], [17], [18]. Electrical signals are associated with wound healing in which the ionic current generated by a wound stimulates epithelial cells to migrate toward the wound center [2]. In fact, electrical stimuli have been shown to override competing stimuli, demonstrating the importance of its role in wound healing [11]. There is evidence to suggest that the progression of a tumor results in a more cathodic environment compared to surrounding tissue, which may provide stimuli for migration of metastatic cells away from the primary tumor [21].

Many studies have investigated migration of cancer cells and found that most cancer cell lines undergo electrotaxis [7], [8], [32], [97], [99]. Among these are lung, breast, and prostate cancer cells. In the breast cancer system, strong electrotaxis is associated with metastatic potential [17]. Many mechanisms for electrotaxis have been proposed for both cancerous and non-transformed cell types. Reactive oxygen species (ROS) have been observed as a mediator for electrotaxis via its activation of Akt and Erk1/2 in glioma cells [205]. However, the mechanism of electrotaxis isn’t observed to be consistent between cell lines. Highly metastatic breast adenocarcinoma cells are observed to lose electrotaxis completely when expression of EGFR is abolished while electrotaxis of lung cancer cells was observed to be EGFR independent [97], [100].

These studies on electrotaxis of cancer cells tend to utilize a conventional format with isolated cancer cells migrating along a two dimensional surface. This format,
however, does not represent several key features of the TMEN. First, in a TMEN, particularly during the early stages of tumor development, cancerous cells are confined in a substantially larger population of non-transformed cells. In this setting, a migrating cancer cell must not only overcome the physical constraints imposed by a crowded environment, but also overcome the cell-cell interactions mediated by surface receptors, such as E-cadherin. While E-cadherin is lost in aggressive cancer cells [206], [207], recent work shows that the loss of E-cadherin is not required for metastasis [208]. Moreover, in the early stages of tumor development, even in instances where E-cadherin expression is ultimately lost, the surface receptor will still be expressed by both the cancerous and non-transformed cells. Thus, the role of cell-cell interactions and the expression of E-cadherin in cancer cell electrotaxis in a heterogeneous, crowded environment remains to be investigated.

Secondly, in breast cancer, the TMEN is fibrillar [154], [209]. Migrating cancer cells are observed to extend along these fibers which radiate from the surface of a tumor [210]. Meanwhile, the tumor-associated electric field extends radially from the tumor and parallel to the fibers. This geometric arrangement poses a potential challenge for electrotaxis of cells because a trademark of electrotaxis is the spreading of cells in the direction orthogonal to the electric field [19], [119]. The spreading orthogonal to the electric field is believed that this process minimizes the voltage drop across the cell body. Since the fibrillar arrangement of the TMEN limits cell spreading in the preferred direction, it is unclear in what way electrotaxis might be affected as a result of geometric constraints of the TMEN.
To examine these questions, an apparatus was developed which reflects the TMEN while providing ease of visualization. Herein, we have adapted a conventional electrotactic assay to incorporate both invasive MDA-MB-231 cells and non-invasive, non-transformed MCF-10A-pLKO breast epithelial cells in co-culture to characterize electric field driven migration in a simple apparatus. We have also perturbed the expression of E-cadherin in the MDA-MB-231 cell line to probe the role of cell-cell interactions during electrotaxis within the TMEN. We then patterned fibronectin onto the surface of the conventional electrotactic assay to constrain non-transformed MCF-10A breast epithelial cells, which are phenotypically homogenous relative to MDA-MB-231 cells (i.e. the physical shape of MCF-10A cells is more likely to be conserved than in MDA-MB-231 cells). In doing so, we probe the influence of cell-cell interactions on the electrotaxis of migrating cancer cells in a crowded environment and test how the fibrillar environment outside a tumor may influence electrotaxis.
6.3 Results

6.3.1 Generation of heterogeneous environment

In order to better reflect the heterogeneous nature of the TMEN, a confluent monolayer of PLKO breast epithelial cells was grown within the previously used electrotactic chamber. Highly invasive breast adenocarcinoma MDA-MB-231 cells were then seeded into this layer. We have previously generated an E-cadherin expressing MDA-MB-231 cell line (231-Ecad) and a control line (231) [211]. As tumors are widely heterogeneous, particularly with respect to E-cadherin expression, and spatially confined, this platform better represents the physiological microenvironment migrating cancer cells may encounter during the early stages of the metastatic cascade.

6.3.2 Electrotaxis within an epithelial layer is slowed

In this apparatus, cell speed of both 231-Ecad and 231 was found to be 0.2 and 0.26 μm/min (Table 3). Despite this low speed, these cells were observed to migrate significantly in the direction of the anode of the electric field. To quantify the directedness with respect to the electric field, Equation 1 was used (Fig. 20). A directedness of 1 indicates movement perfectly aligned to the anode of the electric field and a directedness of -1 indicates movement toward the cathode. Only at 0.51 V/cm, the strongest electric field applied, did 231 cells show higher electrotaxis than 231-Ecad cells (p < 0.05). ANOVA was used to determine that the directedness of both cell types showed a dependence on electric field strength.
Table 3. Directedness of migrating cells and cell speed. Cell speed is listed as parenthetical values and reported in \( \mu \text{m/min} \). Sample sizes are listed in brackets.

<table>
<thead>
<tr>
<th>Electric Field (V/cm)</th>
<th>231</th>
<th>231-Ecad</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.08</td>
<td>-0.06</td>
</tr>
<tr>
<td></td>
<td>(0.21)</td>
<td>(0.23)</td>
</tr>
<tr>
<td></td>
<td>[260]</td>
<td>[419]</td>
</tr>
<tr>
<td>0.13</td>
<td>0.09</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>(0.24)</td>
<td>(0.25)</td>
</tr>
<tr>
<td></td>
<td>[352]</td>
<td>[446]</td>
</tr>
<tr>
<td>0.26</td>
<td>0.11</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>(0.26)</td>
<td>(0.26)</td>
</tr>
<tr>
<td></td>
<td>[781]</td>
<td>[376]</td>
</tr>
<tr>
<td>0.51</td>
<td>.28</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>(0.25)</td>
<td>(0.24)</td>
</tr>
<tr>
<td></td>
<td>[554]</td>
<td>[516]</td>
</tr>
</tbody>
</table>

6.3.3 E-cadherin expression but not electric field results in more persistent movement

In order to assess the forward progression of a cell, persistence was calculated from Equation 2 (Fig. 21). A persistence of 1 indicates movement in a straight line while a persistence of 0 indicates no net movement in any direction. Under no external stimuli, even highly motile cells would be expected to have a persistence value close to 0. E-cadherin expression was found to increase the persistence significantly in the absence of an external electric field. Upon exposure to even the weakest electric field, persistence of parental cells lacking E-cadherin expression increased. ANOVA does not indicate that there is any dependence of persistence on electric field after this point for parental cells. For 231-Ecad cells, no dependence on electric field strength was observed, even between the weakest electric field and the absence of electric field.
6.3.4 Invasive cells maintain motion independently of surrounding epithelial cells

The mean square displacement (MSD) of these cell tracks was calculated in order to determine the degree to which cell movement was ballistic (i.e. the degree to which a cell migration path resembles a straight line). While similar to persistence, the MSD is dependent on the time difference between positions. A cell migrating under a “persistent random walk” mode may be found to have a persistence close to 0 despite ballistic movements at short time intervals. The exponent of a power law fit to MSD (Equation 4) is indicative of ballistic ($\beta = 1$), Brownian ($\beta = 0$), or sub-diffusive ($\beta < 1$) movement.

Figure 20. Directedness as a function of applied electric field strength. Directedness is reported for MDA-MB-231 breast cancer cells at applied electric fields of 0, 0.13, 0.26, and 0.51 V/cm. E-cadherin expression mutations are indicated by color with 231-E-cad cells in blue. Red indicates the control 231 cell line. Data points are normalized to the directedness at the lowest electric field of control 231 cells. Error bars indicate the standard error of the mean.
The data was fit to Equation 4 and the exponents were calculated as a function of electric field (Fig. 22).

If invasive cells are unable to penetrate the confluent monolayer adequately, the resulting cell migration would likely be diffusive in nature. However, 231-Ecad cells showed higher MSD at 0 V/cm than empty vector cells (1.43 and 1.24, respectively), both of which were superdiffusive. Notably, neither cell type was observed to undergo subdiffusive movement, which would be expected if they were positionally locked by the surrounding cell sheet. The MSD exponent for 231 cells increased with electric field strength. Comparatively, it decreased to 1.35 for 231-Ecad upon exposure to the weakest electric field, where it stayed even as electric field was increased. At 0.26 V/cm,
MSD exponent of 231 and 231-Ecad converge, indicating that E-cadherin expression may result in increased invasive potential when a directional stimulus is not present.

![Graph showing mean square displacement of cells under varying electric field strengths.](image)

**Figure 22.** Mean square displacement of cells under varying electric field strengths. Electric fields of 0, 0.13, 0.26, and 0.51 V/cm were applied. E-cadherin expression mutations are indicated by color with 231-E-cad cells in blue. Red indicates the control 231 cell line. Since these are calculated values, error bars represent the 95% confidence interval.

6.3.5 E-cadherin expression affects dynamics of reorientation within a surrounding monolayer

Initial trajectory of cells is expected to be randomly distributed since no directional cue is present. During exposure to the electric field, MDA-MB-231 cells migrate toward the anode [17]. In order to calculate the response time to for turning towards the anode, the cells initially moving towards the cathode were analyzed. Data were fit to an exponential recovery (Equation 6) and a characteristic time was determined (Equation 7).
Interestingly, application of an electric field appears to affect the empty vector and E-cadherin knockdown cells in opposite ways (Fig. 23). At application of 0.51 V/cm, E-cadherin expressing cells were faster to respond than at weaker fields. Data is not reported in the absence of an electric field because there is no driving force for reorientation in that case. Empty vector cells took more time to respond to an electric field as the magnitude of the electric field increased. E-cadherin expressing cells were typically slower than control cells at reorienting, except at 0.51 V/cm, where E-cadherin expressing cells were 3.5-fold faster than control cells.

**Figure 23.** Characteristic recovery 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. Large characteristic recovery times are indicative of slow reorientation to an electric field. Error bars represent the 95% confidence interval on calculated parameters. * indicates statistical significance with p < .001.
6.3.6 Electric field application increases invasion rate through porous membrane

A conventional transwell assay was modified to apply an electric field between the upper and lower chambers. Electrical current of either 0 or 50 μA was applied across the membrane. This current corresponds to an electric field of 0.17 V/cm across the width of the membrane. However, the electric field across a porous membrane may vary widely locally depending on the conditions at each pore. Pore resistance may be increased by blockage by fibers from Matrigel or, more importantly, the passage of a cell through the pore. Therefore, we report only the electrical current applied across the membrane rather than the electric potential.

In the absence of electric current, 14.1 control cells per mm² were observed to cross the transwell membrane (Fig. 24). This number increased 2-fold to 29.5 cells/mm².

**Figure 24.** Transwell invasion rates of metastatic breast cancer cells. Electrical currents of 0 and 50 μA were applied with the anode facing the lower chamber of the transwell chamber. * indicates statistical significant deviation from control 231 (blue bar) calculated from an unpaired t-test with α = 0.05.
with application of electric current. 231-Ecad cells did not cross the membrane as frequently, with only 1.6 cells/mm$^2$ detected. However, upon application of the electric current, invasion rate recovered to 15.3 cells/m$^2$ and was not found to be significantly different than parental cells in the absence of an electric current.

6.3.7 Epithelial cells confined to lines recovered electrotrans

Two possibilities exist which explain the recovery of invasion through a porous membrane. Either the magnitude of the electric field increases within a confined geometry or electric field sensing is enhanced due to spatial constraints. Since voltage increases proportionally to electrical resistance, which is dependent on cross-sectional area, the first hypothesis is likely. In order to investigate the degree to which spatial constraint may affect electrotaxis, electrophoretic redistribution of epidermal growth factor receptor (EGFR) across the membrane of breast epithelial MCF-10A cells was modeled. MCF-10A cells were selected because of their phenotypic regularity compared to metastatic MDA-MB-231 breast cancer cells. Based on work done by Fang et al., the electrophoretic mobility of EGFR within a phospholipid membrane was estimated to be 1.08*10$^{-11}$ cm$^2$/s*mV and the coefficient of diffusion with a phospholipid membrane was estimated to be 8.8*10$^{-11}$ cm$^2$ [117]. Assuming a cell radius of 15 μm and approximately $10^3$ – $10^4$ EGFR molecules per cell, the continuity equation can be solved to satisfy Equation 9:

$$\frac{\partial C}{\partial t} + \frac{mE}{r} \frac{\partial C}{\partial \theta} - \frac{D}{r^2} \frac{\partial^2}{\partial \theta^2}(C_{\theta}) = 0$$

(9)

where C is the surface concentration of EGFR, t is time, r is the cell radius, m is the electrophoretic mobility of EGFR, E is the magnitude of the electric field, D is the
coefficient of diffusion of EGFR, and $\theta$ is the angle on the surface of the cell membrane with respect to the electric field.

Equation 9 was solved for various electric field magnitudes with either unconstrained cells or cells confined to lines (Fig. 25). Physiological electric fields were sufficient to cause a significant redistribution of surface receptors toward one direction of the electric field. Confinement of cells onto a thin line sharpened the distribution of

Figure 25. Redistribution of EGFR within an applied electric field in cells which are either A) restricted to the geometry of a 3 $\mu$m line or B) allowed to extend a full 30 $\mu$m across. The x-axis shows location across the cell perimeter.

Figure 26. Electrotaxis of MCF-10A cells on patterned fibronectin. Either fibronectin was deposited as A) featureless or B) patterned lines thin enough to restrict cell area.
EGFR along the cell perimeter, effectively generated a binary distribution of receptors regardless of the electric field applied.

Cells were then seeded onto either a featureless surface of microcontact printed fibronectin or onto microcontact printed lines of fibronectin. In both cases, Pluronic was used to block cell adhesion in unpatterned regions. Surprisingly, electrotaxis was completely inhibited on a featureless substrate (Fig. 26A). However, on restrictive lines, electrotaxis was partially recovered (Fig. 26B). Electrotactic response for cells confined to patterned lines does not appear to be dependent on electric field strength, consistent with pseudo-binary redistribution of surface receptor signaling.
6.4 Discussion

In this study, we report several characteristics of invasive and non-invasive migratory behavior with regards to E-cadherin expression levels using a highly confined electrotaxis assay. First, E-cadherin expression diminishes the electrotactic response of invasive MDA-MB-231 cells. Secondly, E-cadherin expression tends to increase the speed and persistence of MDA-MB-231 cells although differences in these properties tend to approach zero when an external electric field is applied. The extent to which cell migration speed is hindered when invasive cells are seeded within a confluent epithelial layer has implications about previously reported electrotactic potentials of metastatic cells since this environment is more similar to the environment of a developing tumor. Lastly, application of electric current was observed to supplement the invasive potential of cells crossing a Matrigel coated membrane.

Throughout tumor progression, there are characteristic physiological changes in the tumor microenvironment. For example, it is frequently observed that cancer cells upregulate anaerobic respiration associated pathways due to the hypoxic nature of the tumor microenvironment [82]. In the same sense, tumors generally tend to accumulate negative charge, i.e. becoming more cathodic, than surrounding tissue [21]. Therefore, electrotactic behavior may be a driving force for metastatic behavior. Accordingly, we used modified variants of the highly metastatic breast adenocarcinoma cell line, MDA-MB-231. A common development in the progression of metastatic disease is the downregulation of E-cadherin through the epithelial-mesenchymal transition (EMT) [212]–[214]. Metastatic MDA-MB-231 cells are mesenchymal and lack E-cadherin expression. Moreover, E-cadherin has been implicated in the electrotactic response of
other epithelial cells, such as MDCK I [76]. We therefore hypothesized that E-cadherin loss through the metastatic cascade would promote the electrotactic behavior of invasive cells migrating within a representative tissue. While the electrotaxis of isolated parental MDA-MB-231 has been shown before, the modifications chosen here relate to intercellular interactions. Invasive cells were seeded into a confluent layer of epithelial cells for experimentation. This is, to the best of our knowledge, the first confined electrotactic migration assay reported.

The speed of invasive cells was greatly diminished within this assay. Cell speed was reduced to ~0.25 micron/min, compared to literature values of ~0.8 micron/min [17]. The reduction of speed within this confined environment is likely to be more representative of physiological environments than a standard 2D device. Despite this significant decrease in cell speed, both invasive cell lines displayed electrotaxis. We observe a 1.4-fold higher directedness in 231 cells compared to 231-Ecad cells at the highest electric field. At lower electric fields, no significant differences were observed. While directedness of both cell lines increased significantly with increasing electric field strength up to 0.51 V/cm, absolute directedness values only reached 0.28 and 0.20 for 231 cells and 231-Ecad cells, respectively. Since such a wide range of cancer cells are reported to have strong electrotaxis, it is important to consider the effect of a crowded environment on electrotactic directedness.

Since speed is diminished to such an extent, the dynamics of electrotaxis were analyzed. 231-Ecad cells took 58% longer to align at 0.13 V/cm yet they were nearly 4-fold faster at realigning within an electric field of 0.51 V/cm, taking just 28% of the time required for 231 cells. Cell-cell contact appears to increase the rate at which invasive
cells alter their migratory path. However, this comes as a trade-off for electrotactic potential since 231-Ecad cells showed less directed motility. It is likely that having a higher directedness outweighs faster reorientation as the time-scale of metastatic invasion is several orders of magnitude higher than the time-scale of cellular reorientation. Thus, E-cadherin expression appears to be a factor which increases the invasive potential of a metastatic cell although this increase is lost in the presence of an electric field which is characteristic of tumor progression.

The role of cell-cell contacts in the dynamics of reorientation indicates that E-cadherin expression may result in higher persistence of cells migrating within a crowded environment. Indeed, 231-Ecad cells showed higher levels of persistence and more ballistic movement than 231 cells. However, differences in these properties converged to zero upon application of an electric field. Since application of an electric field is known to trigger elongation of cells in order to minimize the potential difference across their membranes [19], [119], E-cadherin expression is likely to be disturbed by such a reorientation.

On average, 2.8 to 5.4% of the confluent sheet was free space at any given time, as gaps appeared and were filled by neighboring cells. Knockdown of E-cadherin may encourage opportunistic movements since cell-cell junctions are weakened. This, of course, would result in more frequent random changes in movement, thus decreasing the time-shifted auto-correlation compared to cells interacting more strongly with their neighbors. As this is consistent with the non-Brownian migration observed in these invasive cells, we can conclude that E-cadherin mediated force transduction allows the
invasive cells more control over their own motion rather than migrating opportunistically toward free space formed stochastically.

To determine if E-cadherin expression is responsible for changes in migration behavior due non-contact mechanisms, a standard transwell invasion assay was used. Among its many functions, E-cadherin is known to bind to β-catenin, affecting localization [201]. Nuclear β-catenin is known to affect gene expression through its interaction with the TCF/LEF family of transcription factors [215]. A trade-off is observed wherein the electric field supplements the invasive potential of MDA-MB-231 cells. While expression of E-cadherin greatly diminishes the ability of MDA-MB-231 cells to invade through a Matrigel coated transwell membrane, application of electrical current increases the invasion rate. An electric current of 50 μA was sufficient to offset the inhibition due to E-cadherin expression and recover invasive potential. It is possible that the electric microenvironment of a developing tumor plays a significant role in the inducing metastatic invasion by this mechanism.
7. Non-uniform electric field generation for high throughput electrotaxis

In preparation for submission
Lalli, M.L., Wojeski, B., Astagiri, A.R.
7.1 Abstract

Cell migration is an important aspect of many physiological processes such as organogenesis, wound repair, and metastasis. Meanwhile, strategies to direct cell migration are essential in many biomedical applications, such as tissue engineering. Current strategies predominately focus on using gradients of chemical cues to direct cell migration. However, gradients of chemical concentrations can be difficult to maintain over long periods of time and do not typically offer the ability to be manipulated in real time. Gradients in electrical potential offer an appealing alternative to chemical gradients. Electrotaxis, the directed migration of cells in an electric field, is generally more potent than chemotaxis. Furthermore, electric fields can be generated quickly, maintained over long periods of time, and can be altered with ease. To our knowledge, all studies of electrotaxis have investigated the response of cells in a uniform electric field. Herein, we developed a novel electrotactic device which generates a non-uniform electric field. This device enables a spectrum of electric fields to be tested at once. We have used this device to observe the electrotaxis of breast epithelial cells and were able to analyze migration in electric fields of 0.5 – 1.0 V/cm in a single device. As a result, this device provides a novel platform to determine the threshold of stimulus required for generating a physical response from cells. This device opens the door to investigate cell migration toward or away from regions of strong electric fields. We propose that this type of electrotaxis may be physiologically relevant in wound healing.
7.2 Introduction

Nonrandom, spatially-directed cell migration is crucial to many physiologically relevant processes such as wound healing, organogenesis, and the progression of metastatic disease [67], [74], [216]. While significant attention is given to directed cell migration in response to spatial gradients in chemical cues [38], [217], [218], it is evident that other stimuli such as substrate stiffness, adsorbed chemical factors, asymmetric geometric patterns, and electric fields are also effective in driving cell migration [30], [42], [43], [96], [159], [219].

Among the many cues that mediate directed cell migration, electric fields are unique in several ways. From the perspective of applications such as tissue engineering, wherein it is desirable to control and tune the direction of cell migration, electric fields are easy to impose and modulate. For example, while complex devices are required to generate an alternating chemical field [220], an alternating electric field can be created by simply connecting the electrodes to a source of alternating current. Moreover, electric fields can be generated in a stable fashion over long periods of time compared to diffusion-dependent chemical gradients which may not be continuously stable over several hours without the use of external pumps [221], [222].

Extracellular electric fields are associated with both healthy and diseased physiology. Transepithelial potentials (TEPs) on the order of 10 mV have been recorded in ductal tissues such as the skin, breast and prostate [2], [17], [18]. Larger potentials result from the electrokinetic flow of blood in circulation [19]. In the tumor microenvironment, rapid cell growth and phenotypical changes in cell surface charge create an electric field between the tumor and the adjacent tissue [21]. While electric
fields are prevalent in numerous physiological contexts, their role in guiding cell migration is poorly understood relative to other guidance cues: a cursory literature search reveals that nearly 300 times as many scientific papers mention chemotaxis compared to those which mention electrotaxis.

A wounded epithelial layer in the body generates electrical currents which stimulate wound closure [125], [223]. The application of external electric fields has been implemented therapeutically for closure of chronic wounds such as ulcers. The gut microbiome may be exoelectrogenic, using electric fields to attract an immune response for wounds in the intestinal epithelia [22]. Immune cells are involved with wound healing in a number of ways such as the local secretion of growth factors [224]. We hypothesize that in order for immune cells to be attracted to wound sites based on sensing of electric fields, certain cells must be capable of not only sensing the presence of an electric field, but also the direction of greater field strength, and migrating toward it. This mode of migration could be described as orthogonal electrotaxis.

Some work has shown that breast cancer cells will predominately undergo chemotaxis in the non-linear concentration gradient compared to a linear gradient with the same limits [59]. In diffusion dependent chemical gradient generators such as the “Christmas tree,” the chemical gradient and its derivative exist in the same plane, making it is difficult to distinguish between migration directed toward higher chemokine concentration or toward a region of higher concentration gradient. The direction of a non-uniform electric field and its derivative are orthogonal to each other, which helps distinguish between migration directed by the electric field primarily or directed by a gradient in the strength of the electric field.
In electrodeposition, a Hull chamber is commonly used to expose a metallic surface to a linear gradient of electric fields by aligning the electrodes at nonparallel angles to one another. The same principle was applied here with the goal of generating a linear gradient in electric field magnitude which could be exposed to adherent cells. While other geometric configurations could be designed to yield more complex, non-linear electric field gradients, we sought to demonstrate a linear gradient in electric field magnitude as a proof-of-concept. Herein, we describe a novel device which creates a non-uniform electric field with a linear gradient in electric field strength, analogous to an electrochemical Hull cell. Additionally, with minor alterations to the geometry of the device or by modifying the source of the electric current, virtually any desired electric field could be generated. Since a spectrum of electric fields can be tested simultaneously, this device increases the accessibility of high throughput testing of electrotaxis. This low-cost device can be fabricated easily without advanced microfluidic equipment and can be implemented in a variety of methods.
7.3 Results and Discussion

7.3.1 Computational model for designing a device to generate non-uniform electric fields

In order to design a suitable device to generate the desired electric field, we modeled the differential form of Gauss’s law (Equation 1) using the partial differential equation toolbox in MATLAB. To translate the principle of the Hull cell to this cell migration device, a simple linear gradient in electric field strength was desired. A voltage of 1 V was modeled across the electrodes, treating the electrodes as uniform in potential and the remainder of the device as electrically insulating.

The model predicted that arranging the electrodes at a 45° angle to each other would result in the generation of a non-uniform electric field with an approximately linear gradient in electric field (Fig. 27). The electric field ranged from its greatest magnitude where the electrodes are closest to its weakest point where the electrodes are maximally distant from each other. The largest magnitude of the electric field was approximately double that of the smallest (Fig. 27B). The distance between the electrodes at the point of weakest field strength was double that between the electrodes at the point of greatest field strength. Therefore, the lower bound of the range of electric fields capable of being generated by this device at any given time point will be half the upper bound.
Since electric field lines are perpendicular to the surface of the electrodes (Fig. 28), the lines of equipotential are non-parallel between two non-parallel electrodes (Fig. 27A). At any position relative to the electrode surfaces, the axis of equipotential and the axis of electric field can be determined. Since these axes will be perpendicular to one
another, displacement of particles or cells can be decomposed into the component parallel with the electric field and the component parallel with the electric field gradient.

### 7.3.2 Device construction

Based on the model predictions, a proof-of-concept device with one electrode positioned at 45° to the other was constructed. The fabrication process is described in Figure 29. An electron beam evaporator was used to deposit gold electrodes onto the surface of glass. Although successful, the resultant electrodes were brittle when deposited on a hard surface and would break during construction of the cell chamber (Fig. 29D-F). To prevent electrode breaking, PDMS was chosen as a softer substrate. Rather than
depositing the electrodes, they were simply allowed to cure in plane with the PDMS prepolymer. Since the electrodes are in the same plane as the PDMS surface, when a DC3140 wall is built up around the space between electrodes, current can pass from outside of the chamber into the chamber without needing to expose the cell culture medium to the atmosphere.

7.3.3 Device performance

Fully constructed devices were tested initially without cells in the chamber. No generation of electrode products was observed when voltages less than the standard potential of hydrolysis were used. At voltages higher than the standard potential of

Figure 29. Preparation of the non-uniform electric field generator. A) A PDMS block is partially cured in a polystyrene dish without any features added. B) Prefabricated aluminum electrodes are placed onto the surface of the partially cured PDMS. At this stage, convection currents are not present to cause electrode placement to change. C) Once cured, the PDMS is cut to fit the size of a glass microscope size. D) A wall of DC 3140 silicon adhesive is constructed in order to define a cell adhesion area. E) A glass roof is placed over that area in order to enclose the device chamber. F) A potentiostat or power supply is connected to the electrodes in order to close the circuit.
hydrolysis, hydrogen and oxygen were generated at the electrodes in gaseous form, which displaces media from the cell adhesion area. This puts an upper limit on the maximum electric field that the device can generate which would be the standard potential of water divided by the shortest path between the electrodes. Over the course of 6 hours, the devices were stable during application of 0.5 V to the electrodes. At this potential, the electric field generated in the device ranges from 0.5 V/cm at the weakest region to 1.0 V/cm at the opposite end. The voltage drop across the electrodes was validated with a standard voltmeter.

MCF-10A cells were left to adhere within the device and then imaged. Cell imaging was performed at 5x magnification due to the location of viable focal planes. Since the PDMS alone is flexible, it must be set onto a glass slide in order to prevent mechanical instability during experimentation. When mounted on a glass slide for mechanical stability, the distance at which the focal plane of the microscope lens intersects the device is a concern. On a Zeiss AxioVert 200M microscope, it was possible to image at 5x magnification but not at higher magnification as a result.

### 7.3.4 Cell viability

Having constructed the device and validated the electric field generated, we next tested cell viability within the device during application of an electric field. MCF-10A breast epithelial cells were exposed to a gradient of electric fields from 0.5 to 1.0 V/cm. Products of electrolysis can be detrimental to the health of cells. At the very least, evolution of gaseous compounds will displace cell culture media, starving and dehydrating cells. Often, electrodes are separated from cell culture chambers during electrotaxis experiments using agar bridges. The use of agar bridges prevents the
diffusion of electrolytic products, such as gaseous hydrogen and oxygen or metallic salts, into the cell culture region. By maintaining low voltages, the generation of such compounds was prevented. Cells remained viable throughout the 6 hour window of observation and continued migrating. Had electrolytic compounds been generated in an appreciable amount, cell migration would likely have ceased.
7.4 Conclusions

Here, we have described a multifunctional and inexpensive device for studying electrotaxis over a spectrum of electric field strengths. Using inexpensive materials, this device was designed and constructed to generate a non-uniform electric field with a linear gradient to study electrotaxis. A range of electric fields from 0.5 to 1.0 V/cm were generated although the upper and lower voltages are not constrained to those values. The upper bound of electric field strength is governed by the standard potential of hydrolysis, which is 1.23 V for water but may vary based on media composition, and the geometry of the electrodes. Using the method described here, any number of electrode geometries can be implemented in order to produce nonlinear gradients in electric field strength. Using this device, one can investigate electrotaxis in a spectrum of electric fields simultaneously, which opens the door to high-throughput analysis. Additionally, many tissue engineering applications can be imagined due to the fact that two axes of stimuli can be taken advantage of.

We believe that this new device is promising for future research that requires the simultaneous exposure of various electric fields. Notably, we have previously described a threshold-dependent electrotactic response in epithelial cells, which could easily be detected using this device. Moreover, the possibility of studying migration toward a region of high electric field magnitude is made possible by this device. We propose orthogonal electrotaxis, migration toward regions of high electric field magnitude, as a physiologically relevant phenomenon and believe future studies need to be performed in this region.
8. Conclusions and Recommendations for Future Work

The goal of this thesis has been to critically and quantitatively analyze how electrotaxis can be utilized in the guidance of epithelial cells. Electric fields are found nearly ubiquitously throughout the body and are believed to be associated with the developing tumor environment [21]. It is well documented that electric fields are a more potent director of cell migration than chemical cues [11]. Moreover, *in vitro*, electric fields are simpler and more versatile to establish than concentration gradients. As a result, the utilization of electrotaxis is promising in the future of tissue engineering. For both tissue engineering applications and for developing novel cancer therapies, a thorough understanding of electrotaxis in human cells is necessary. In order to reach the level of quantitative analysis desired, we developed various platforms and created a collection of analytical tools.

Through the course of this thesis we have investigated several properties of electrotaxis, particularly among the MCF-10A, non-transformed breast epithelial cell line. We found that MCF-10A cells migrate toward the anode of an electric field. Their directed migratory response is dependent on the magnitude of the applied electric field both in terms of the magnitude of the response, but also in terms of an electric field magnitude threshold. While isolated cells migrating individually require a threshold of at least 0.26 V/cm, the same cells when in contact with each other lose that threshold requirement. However, there comes a trade-off wherein the response threshold is removed, but response time increases for cells within clusters. Whether this is a result of leader cell dynamics or if it is caused by the fact that cells in a crowded environment are limited in directional selection for migration is unclear. However, from a tissue
engineering perspective, we see an insight to controlling cell behavior differentially based on the local characteristics of the cellular environment.

The observation that clustered epithelial cells had no distinct threshold for sensing and responding to an applied electric field raises a number of questions. Specifically, there must be a pathway related to electric field sensing that is dependent on cell-cell interactions or changes associated with a clustered environment. Therefore, we next looked into breaking down the mechanism which leads clustered cells to have this more vigorous response to electric field application. In order to do this, we had to separate the factors associated with cell clustering. These factors are 1) cell-cell interactions, mediated in a large part by E-cadherin and 2) the spatial confinement associated with cells crowding each other. Investigation of both these aspects separately is required because crowding within a cell cluster presents both biophysical and biochemical differences from the isolated cell. Since the mechanism of electrotactic sensing and response is largely unknown, we cannot predict whether physically altering cell shape during crowding, which results in a different interaction of an applied voltage, would be more or less influential than cell-cell communication and signaling.

Cell migration assays are dependent on the ability to visualize the cells as they move and record their migration paths. As a result, analysis of cell migration is highly labor intensive. To circumvent this, a new cell tracking algorithm had to be developed. Initially, the ImageJ platform for cell tracking was utilized, but was found to be cumbersome. We wrote a manual cell tracking GUI in MATLAB to improve this process. However, tracking of a single cell still took upwards of 1 minute using this approach. There have been many developments in automating cell tracking, such as TrackMate
[186] and the MOSAIC Suite [187], which are compatible with Fiji. Typically, tracking methods such as these require the use of fluorescent labeling steps, which add experimental difficulty.

We developed an algorithm which increased the throughput of our experimental analysis by 5-fold without requiring additional labeling steps. While automated cell tracking is not a new development, tracking of clustered cells without fluorescent labeling still remains a challenge. Despite significant efforts to develop algorithms for tracking cells using phase-contrast imaging alone, it remains difficult to identify cell boundaries computationally. We overcame this challenge in part by allowing the algorithm to partially analyze a frame in order to focus on a single cell. By cropping an image around a single cell, image analysis techniques are enhanced greatly and can be applied to images which may be out of focus in some areas. Once a cell is identified in a single frame, the algorithm uses that location as a guess for the next frame and sequentially repeats the process. In this way, rather than analyzing each frame in its entirety, the algorithm processes each frame multiple times but only analyzing a small region during any pass. A second development in our approach is to estimate the derivative of pixel intensity at any given point. Because the inside of the cell is filled with organelles and vacuoles, the derivate of pixel intensity is generally higher in these regions. This helps distinguish adjacent cells from one another even when highly crowded. We hope that this method will be adopted by others researching cell tracking because it could be easily combined with other approaches, particularly machine learning methods for cell identification, in order to produce an even more robust method.
When we knocked down E-cadherin expression and used this algorithm to process large sample sizes of data, we found that electrotaxis was only mildly inhibited in clustered cells. E-cadherin was only knocked down by 60% in order to ensure that intercellular bonds could continue to be formed so that cell clusters would not disseminate. In this way, the physical crowding of the cell cluster is maintained but cell-cell interactions are weakened. Notably, the electrotactic responses of isolated cells were disrupted even though E-cadherin mediated intercellular interactions are not present. Isolated cells with lowered E-cadherin expression where faster to respond to electric fields but, at intermediate electric field strengths, were less directed in their migration toward the anode. Therefore, E-cadherin likely plays a broader role in electrotaxis beyond mediation of cell-cell interactions. E-cadherin is known to bind to β-catenin which, if left unbound, will localize toward the nucleus [201]. In cancer progression, E-cadherin expression is typically lost and β-catenin mediates expression of several oncogenes [158], [89]. We believe that the data presented here is in agreement with the nature of E-cadherin expression and cancer progression since loss of E-cadherin expression results in faster response time to electric fields in isolated cells.

Since the TMEN is electrically active, both a strong electrotactic response and fast orientation times to an electric field may play important roles in metastasis. To investigate this, we focused on the ability of metastatic cells to migrate in an applied electric field. Many studies have shown that a strong electrotactic response correlates with high metastatic potential [17]. However, cancer cell migration within the TMEN is very different from single cell migration in a standard migration assay [225]. To better replicate the TMEN, we developed an assay where metastatic cells are forced to migrate
through a confluent layer of non-metastatic epithelial cells. Although not perfectly replicative of the TMEN in vivo, this assay has a number of benefits. Namely, we can alter the E-cadherin expression of the metastatic cells to determine the interaction between E-cadherin and electrotaxis in cancer cells. Secondly, the crowded nature of the TMEN is emulated, so despite the fact that cells which have undergone EMT do not tend to form stable adherens junctions in vitro, we are able to sterically hinder the cells. This is important because in vitro, cells align orthogonally to the electric field in order to minimize the voltage drop across the cell membrane [94], [127]. In vivo, cells are typically confined to the extracellular matrix, which is fibrillar in nature. It is therefore highly unlikely that assays which allow metastatic cells to align orthogonally to the electric field can be taken as representative of what occurs within the body.

Using this new assay, we tested the ability of metastatic breast cancer MDA-MB-231 cells to invade through a confluent layer of MCF-10A cells. We varied the E-cadherin expression level of the MDA-MB-231 cells so that the wild type cell line had no E-cadherin expression and the experimental cell line had roughly 10% E-cadherin expression as wild type MCF-10A cells [156]. We found that E-cadherin expression reduced the overall electrotactic response observed in the MDA-MB-231 system. Initially, this was surprising because reduction of E-cadherin expression in MCF-10A cells resulted in a decrease of electrotaxis while the opposite occurred in the invasive cells. It is entirely possible that E-cadherin plays different roles in the two cell types, even with respect to electrotaxis. For instance, in epithelial wound healing, electric fields provide an overriding stimulus for healing [11]. Presumably, since it is important for epithelial cells, which natively express E-cadherin, to sense and respond to those fields,
E-cadherin or E-cadherin mediated cell-cell bonds help to enhance this response. Comparatively, E-cadherin expression is lost in cells during cancer progression, but responding to electric fields may still be important for invasive cells to disseminate from a primary tumor. Therefore, E-cadherin expression may retard electrotaxis in invasive cells, possibly by encouraging heterogeneous cell-cell interactions, and its loss is consistent with a more metastatic phenotype.

We then modified a more standard invasion assay by applying an electric field across a permeable membrane coated with Matrigel ECM. Control cells were able to migrate from the upper chamber, down through the Matrigel layer, and into the bottom chamber of the transwell assay. We found that MDA-MB-231 cells with added E-cadherin expression would not invade through the membrane, which had pores of 8.0 microns. However, if a weak electric field was applied across the membrane through two custom made agar bridges, invasion through the membrane was rescued. When the electric field was applied with control cells migrating, the number of cells able to invade through the membrane increased. Because there are no other cells present, it is clear that the effect of E-cadherin on retarding invasion of MDA-MB-231 cells is not limited to its role in cell-cell interactions. It is possible that E-cadherin expression pushes a metastatic cell toward a less invasive phenotype by binding with β-catenin and reducing the expression of certain oncogenes. However, we see that the presence of an electric field increases the invasive properties of these cells.

A major difference between the transwell assay and the two dimensional invasion assay is that the cells migrating through the pores are spatially restricted except in the direction of the electric field lines. As previously mentioned, in the presence of an
electric field, cells have the tendency to spread orthogonally to the field in order to minimize the voltage drop across their cell membranes. We hypothesized that restricting cells in the directions orthogonal to the electric field would enhance their electrotaxis. One possibility that cell shape restriction may lead to enhanced electrotaxis results from redistribution of surface bound cell receptors along the cell membrane due to electromigration. To this effect, we modeled the surface concentration of EGFR along the membrane of MCF-10A cells under an applied electric field of various strengths. Our model found that EGFR molecules, as shown by others experimentally, predominately localizes toward the side of the cell facing the cathode [117]. Size restriction only narrowed the distribution of these molecules. These results are in agreement with the hypothesis that electrotaxis itself might be a subset of chemotaxis, where the cell may sense a chemical concentration gradient as a result of receptor distribution [226].

To test this, we used microcontact patterning [227] to produce lines of fibronectin within the electrotactic chamber. To compare against simulated data, we seeded MCF-10A cells onto these lines and allowed them to migrate within the electric field. As a control, a featureless block of fibronectin was deposited onto the surface of the electrotactic devices. Surprisingly, when adherent to fibronectin deposited in this way, MCF-10A cells did not undergo electrotaxis. This may be a result of a number of factors, such as poor orientation of adhesion sites due to the protein transfer method. Importantly, when fibronectin lines were deposited, electrotaxis in MCF-10A cells was rescued. This fibrillar environment, which is more representative of the breast TMEN compared to a planar substrate, provides some insight into how electrotaxis may be affected by the environmental constraints imposed on cells in vivo.
In all experiments performed using the two dimensional electrotactic devices, including those which had fibronectin lines incorporated into them, at least four electric field strengths were used in order to gain insight into trends. In Chapter 4, a threshold of electrotactic response for isolated cells was observed but then disappeared for cells within clusters. In order to increase the throughput of future experiments, we sought to develop a method for exposing cells to a spectrum of electric fields simultaneously. Inspiration was taken from the Hull cell, a device typically used in electrochemistry in order to observe the electrodeposition of metals at various electric fields simultaneously. The Hull cell relies upon angled electrodes so that lines of equipotential are nonparallel, therefore generating a stronger electric field where the electrodes are closest and a weaker electric field where they are farthest apart. We simulated, fabricated, and validated this platform in a cell compatible manner. In addition to testing a range of electric fields simultaneously, this device opens the door for investigating migration in the direction of the gradient of electric field strength, which may be physiologically important for the immune response.

In summary, we have investigated the role of the cellular environment on electrotaxis, including modulation of electrotaxis through cell-cell interactions or through geometric constriction. We found that cell clustering enhances electrotaxis and proceeded to show that both intercellular interactions mediated through E-cadherin and the crowding of the environment played important roles in electrotaxis. In doing so, we developed a novel tracking algorithm to increase the throughput of experiments. Finally, we produced a novel device which allows future researchers to test a wide range of electric fields simultaneously. Future directions should focus on a mechanistic approach
to studying electrotaxis using the platforms developed here. Additionally, experiments on
electrotaxis in cancer cells generated by featureless two dimensional migration assays
should be replaced when possible with assays which incorporate a featured surface. We
now have reason to suspect that the fibrillar environment associated with the breast
TMEN may work synergistically with the electroenvironment to promote metastasis
through an electrotaxis. Mechanistic research into inhibiting electrotaxis of invasive cells
using these platforms may have potential more broadly by providing targets for novel
chemical therapeutics.
9. References


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[71] X. Trepat, M. R. Wasserman, T. E. Angelini, E. Millet, D. a. Weitz, J. P. Butler,


10. Nomenclature

**TMEN**: Tumor microenvironment, the collection of matrix proteins and supporting cells which surround and provide strength to the tumor.

**VEGF**: Vascular endothelial growth factor, a growth hormone which stimulates growth and the formation of new blood vessels.

**TEP**: Transepithelial potential, the voltage difference across a layer of epithelial tissue.

**ECM**: Extracellular matrix, structural proteins that comprise the space between tissues and support cells.

**MTOC**: Microtubule organizing center, a region within the cell from which cytoskeletal proteins emerge and transduce force.

**EGF**: Epidermal growth factor, a molecule which is used in chemical signaling to stimulate growth and migration in certain cell types including epithelial cells.

**EGFR**: EGF receptor, a surface bound receptor which binds with EGF and transduces the signal into the cell by activating chemical pathways.

**VGSC**: Voltage gated sodium channel, a membrane bound pump which controls the flow of sodium ions into and out of the cell.

**THG**: Third harmonic generation, a multiphoton imaging technique which is used to observe fluids next to cell membranes.

**ROI**: Region of interest, areas within an image that are of import to the user.
11. Appendices
11.1 Appendix A: Automated Cell Tracker

11.1.1 GUI

![User interface for automated cell tracking.](image)

**Figure S1.** User interface for automated cell tracking.

11.1.2 Code

```matlab
function varargout = cell_motility_auto(varargin)

% CELL_MOTILITY_AUTO M-file for cell_motility_auto.fig
% CELL_MOTILITY_AUTO, by itself, creates a new CELL_MOTILITY_AUTO
% or raises the existing singleton
% H = CELL_MOTILITY_AUTO returns the handle to a new
% CELL_MOTILITY_AUTO or the handle to
% the existing singleton
% CELL_MOTILITY_AUTO('Property','Value',...) creates a new
% CELL_MOTILITY_AUTO using the
% given property value pairs. Unrecognized properties are passed
% via
% varargin to cell_motility_auto_OpeningFcn. This calling syntax
% produces a
% warning when there is an existing singleton
% CELL_MOTILITY_AUTO('CALLBACK') and
% CELL_MOTILITY_AUTO('CALLBACK',hObject, ...) call the
% local function named CALLBACK in CELL_MOTILITY_AUTO.M with the
% given input
% arguments.
```

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% *See GUI Options on GUIDE's Tools menu. Choose "GUI allows only one instance to run (singleton)".*
% See also: GUIDE, GUIDATA, GUIDATA

% Edit the above text to modify the response to help cell_motility_auto

% Last Modified by GUIDE v2.5 12-Jan-2015 16:21:54

% Begin initialization code - DO NOT EDIT
gui_Singleton = 1;
gui_State = struct('gui_Name', mfilename, ...
'gui_Singleton', gui_Singleton, ...
'gui_OpeningFcn', @cell_motility_auto_OpeningFcn, ...
'gui_OutputFcn', @cell_motility_auto_OutputFcn, ...
'gui_LayoutFcn', [], ...
'gui_Callback', []);
if nargin && ischar(varargin{1})
    gui_State.gui_Callback = str2func(varargin{1});
end
if nargout
    [varargout{1:nargout}] = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end
% End initialization code - DO NOT EDIT

% --- Executes just before cell_motility_auto is made visible.
function cell_motility_auto_OpeningFcn(hObject, eventdata, handles, varargin)
% This function has no output args, see OutputFcn.
% hObject    handle to figure
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
% varargin   unrecognized PropertyName/PropertyValue pairs from the command line (see VARARGIN)

% Choose default command line output for cell_motility_auto
handles.output = hObject;

% Update handles structure
guidata(hObject, handles);

% UIWAIT makes cell_motility_auto wait for user response (see UIRESUME)
% uiwait(handles.figure1);

% --- Outputs from this function are returned to the command line.
function varargout = cell_motility_auto_OutputFcn(hObject, eventdata, handles)
% varargout  cell array for returning output args (see VARARGOUT);
function edit1_Callback(hObject, eventdata, handles)
% hObject    handle to edit1 (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'String') returns contents of edit1 as text
%        str2double(get(hObject,'String')) returns contents of edit1 as a double

Axes(handles.axes1);
cla;
filename = get(handles.edit1, 'String');
handles.filename = filename;
mov = aviread(filename);
imshow(mov(1).cdata)
t = length(mov);
coun = 0; a = 0;
xloc = zeros(1,200); yloc = xloc;
[ylimm xlim z] = size(mov(1).cdata);
handles.t = t; handles.coun = coun;
handles.xloc = xloc; handles.yloc = yloc;
handles.ylimm = ylimm; handles.xlim = xlim; handles.z = z;
handles.mov = mov;
c = 0; handles.c = c;
trk = 0; handles.trk = trk;
handles.auto = 0;
handles.manual = 0;
guidata(hObject,handles)

% --- Executes on button press in checkbox1.
function checkbox1_Callback(hObject, eventdata, handles)
% hObject    handle to checkbox1 (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hint: get(hObject,'Value') returns toggle state of checkbox1

% --- Executes on button press in pushbutton2.
function pushbutton2_Callback(hObject, eventdata, handles)
% hObject    handle to pushbutton2 (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
xloc = handles.xloc; yloc = handles.yloc;
coun = handles.coun;
coun = coun + 1;
axes(handles.axes1);
hold on
[xloc(coun) yloc(coun)] = ginput(1);
plot(xloc, yloc, 'or', 'Linewidth', 2)
handles.coun = coun;
handles.xloc = xloc; handles.yloc = yloc;
track = struct('Location',cell(1));
tracker = repmat(track,coun,1);
s = struct('start',cell(1),'track',cell(1));
databank = repmat(s,coun,1);
handles.tracker = tracker;
handles.databank = databank;
guidata(hObject,handles)

% --- Executes on button press in pushbutton3.
function pushbutton3_Callback(hObject, eventdata, handles)
% hObject    handle to pushbutton3 (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
c = handles.c;
autocounter = 0;
manualcounter = 0;
coun = handles.coun;
xloc = handles.xloc;
yloc = handles.yloc;
ylimm = handles.ylimm;
xlim = handles.xlim;
trk = handles.trk;
t = handles.t;
mov = handles.mov;
databank = handles.databank;
c = c + 1;
axes(handles.axes3);
cla;
if c == coun
    bar(100,'g'),ylim([0,100])
else
    bar(c/coun*100,'b'),ylim([0,100])
end

trk = trk + 1;
databank(trk).start = [xloc(c), yloc(c)];
databank(trk).track = zeros(t,2);

yy = floor(yloc(c));
xx = floor(xloc(c));
axes(handles.axes2);
cla;

for j = 1:t
    if yy < 101 || xx < 101 || yy + 101 > ylimm || xx + 101 > xlim
        break
    end

    cc = mov(j).cdata((yy-100):(yy+100),(xx-100):(xx+100),2);
drawnow
    aa = cc;
    aa(101,:) = 1; aa(:,101) = 1;
    imshow(aa)

    cc = double(cc);
    cc = cc/max(max(cc));
    cc = log(cc);
    cc = -cc;
    cc(cc == Inf) = 0;
    cc = cc/max(max(cc));
    down = cc(3:201,2:200);
    up = cc(1:199,2:200);
    left = cc(2:200,1:199);
    right = cc(2:200,3:201);
    ul = cc(1:199,1:199);
    ur = cc(1:199,3:201);
    ll = cc(3:201,1:199);
    lr = cc(3:201,3:201);
    diff1 = abs(right - left);
    diff2 = abs(up - down);
    diff3 = abs(ul - lr);
    diff4 = abs(ur - ll);
    difference_matrix = (diff1 + diff2 + diff3 + diff4);
dm = double(difference_matrix);
dm = dm/max(max(dm));
    mask1 = im2bw(cc);
    ccl = cc.*mask1;
    cc2 = imerode(ccl,strel('disk',1));
    aa = zeros(size(cc2));
    aa(2:200,2:200) = dm;
    dme = cc2.*aa;
    dmf = im2bw(dme,graythresh(dme(dme < max(max(dme)))));
    dmg = imdilate(dmf,strel('disk',1));
    dmh = imerode(dmg,strel('disk',2));
    bwl = bwlabel(dmh);
    hh = regionprops(bwl);
    i = ismember(bwl, find([hh.Area] >= 50));
    filledup = imfill(i,'holes');
    bwl = bwlabel(filledup);
}
hh = regionprops(bwl,'Centroid');
hh = [hh.Centroid];
xs = hh(1:2:length(hh)-1);
ys = hh(2:2:length(hh));
distim = sqrt((101-xs).^2+(101-ys).^2);
index = find(distim == min(distim));
if min(distim) > 10
    [hm mh] = ginput(1);
    info2.Centroid(1) = round(hm);
    info2.Centroid(2) = round(mh);
    manualcounter = manualcounter + 1;
else
    try
        info2.Centroid(1) = round(xs(index));
        info2.Centroid(2) = round(ys(index));
        autocounter = autocounter + 1;
    catch
        [hm mh] = ginput(1);
        info2.Centroid(1) = round(hm);
        info2.Centroid(2) = round(mh);
        manualcounter = manualcounter + 1;
    end
end
yy = floor(info2.Centroid(2) + yy-101);
xx = floor(info2.Centroid(1) + xx-101);
if yy < 101 || xx < 101 || yy + 101 > ylim || xx + 101 > xlim
    break
end
databank(trk).track(j,:) = info2.Centroid;
end
handles.databank = databank;
databank(trk).track;
handles.c = c;
handles.trk = trk;
handles.autocounter = autocounter;
handles.manualcounter = manualcounter;
guida(hObject,handles)

% --- Executes on button press in pushbutton4.
function pushbutton4_Callback(hObject, eventdata, handles)
% hObject    handle to pushbutton4 (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
trk = handles.trk;
auto = handles.auto;
manual = handles.manual;
t = handles.t;
print_array = NaN(trk*t,5);
track_num = 0;
databank = handles.databank;
filename = handles.filename;
for i = 1:trk
    for j = 1:t
        if databank(i).track(j,1) == 0 || databank(i).track(j,2) == 0
            break
        end
    end
end
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end
track_num = track_num + 1;
if j == 1
    print_array(track_num,:) = [track_num, i, j, ...
databank(i).start+databank(i).track(j,:)-101];
else
    print_array(track_num,:) = [track_num, i, j, ...
    print_array(track_num-1,4:5)+databank(i).track(j,:)-
    101];
end
end
end

nam = filename;
xlswrite(nam, cell(java.lang.String(nam)), 'A1:A1')
xlswrite(nam, cell(java.lang.String('Type')), 'D1:D1')
a = get(handles.popupmenu2,'Value');
switch a
    case 2
        xlswrite(nam, cell(java.lang.String('Single Cells')), 'E1:E1')
    case 3
        xlswrite(nam, cell(java.lang.String('Clusters')), 'E1:E1')
end
strArray = java_array('java.lang.String', 8);
strArray(1) = java.lang.String('Data
Point');
strArray(2) = java.lang.String('Track');
strArray(3) = java.lang.String('Frame');
strArray(4) = java.lang.String('X');
strArray(5) = java.lang.String('Y');
strArray(6) = java.lang.String('Distance');
strArray(7) = java.lang.String('Velocity');
strArray(8) = java.lang.String('Pix Val');
cellArray = cell(strArray)';
xlswrite(nam, cellArray, 'A2:H2')
string = sprintf('A3:H%d',track_num+2);
xlswrite(nam, print_array, string)
xlswrite(nam, [auto, manual, auto/(auto+manual)], 'G1:I1')

% --- Executes on button press in pushbutton5.
function pushbutton5_Callback(hObject, eventdata, handles)
% hObject    handle to pushbutton5 (see GCBO)
% eventdata  reserved             - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
drawnow
trk = handles.trk;
auto = handles.auto;
manual = handles.manual;
autocounter = handles.autocounter;
manualcounter = handles.manualcounter;
b = get(handles.checkbox1,'Value');
if b == 0
    trk = trk - 1;
else
    auto = auto + autocounter;
    manual = manual + manualcounter;
end
handles.auto = auto;
handles.manual = manual;
handles.trk = trk;
guidata(hObject,handles)

% --- Executes on selection change in popupmenu2.
function popupmenu2_Callback(hObject, eventdata, handles)
% hObject    handle to popupmenu2 (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: contents = cellstr(get(hObject,'String')) returns popupmenu2 contents as cell array
% contents{get(hObject,'Value')} returns selected item from popupmenu2

% --- Executes during object creation, after setting all properties.
function popupmenu2_CreateFcn(hObject, eventdata, handles)
% hObject    handle to popupmenu2 (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcns called

% Hint: popupmenu controls usually have a white background on Windows.
% See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end
11.2 Appendix B: Dissertation Committee Approval

NORTHEASTERN UNIVERSITY
Department of Chemical Engineering
Dissertation Committee Approval Form

Student: Mark Lall

Research Topic: Electrodes in Wavy Epithelium and Bicuspid Eustachian

Committee Agreement (Minimum requirement 4 members; expand if needed):

Committee Member: Name (typed) Edgar D. Gough
Signature: __________________________ Date: 2/25/14

Committee Member: Name (typed) William Tunn
Signature: __________________________ Date: 2/25/14

Committee Member: Name (typed) Erin J. Cram
Signature: __________________________ Date: 2/25/14

Committee Member: Name (typed)
Signature: __________________________ Date: ______

Dissertation Advisor: Name (typed) Anand Asthana
Signature: __________________________ Date: 2/25/14

Received by Administrative Assistant in the Chemical Engineering Main Office:

Administrative Assistant: Patricia Rowe Date: 2/24/14
Chemical Engineering Main Office

Graduate Coordinator Approval: __________________________ Date: 2/25/14

An electronic copy of fully signed form to be maintained in the Chemical Engineering Main Office and provided to the student and the student’s advisor.
11.3 Appendix C: Dissertation Proposal Approval

NORTHEASTERN UNIVERSITY

Department of Chemical Engineering
Dissertation Proposal Approval Form

Dissertation Proposal Title: Targeting dynamic migratory response in ovarian, bladder, and prostate cancer cells

Author: Mark Lee

Proposal Approval:

Committee Member: Edgar E. Smith Date: 4/12/2014
Committee Member: Chin Ho Lee Date: 4/16/2014
Committee Member: Rosemary Banks Date: 4/21/2014
Committee Member: Date: 

Dissertation Advisor: Date: 4/15/2014

Received by Administrative Assistant in the Chemical Engineering Main Office:

Administrative Assistant: Patricia Rowe Date: 4/14/2014

Chemical Engineering Main Office

An electronic copy of fully signed form to be maintained in the Chemical Engineering Main Office and provided to the student and the student’s advisor.