Physical and Molecular Characterizations of Galvanotaxis and Its Implication in Glioblastoma

by

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Abstract

In order for the machineries within a living organism to work properly, various types of signals must spatially and temporally orchestrate in harmony. However, aside from chemotaxis and action potentials, little is known about the physiological relevance of endogenous electric fields. These steady and long-lasting direct current electric fields (dcEFs) are fundamental in guiding cell migration, a phenomena termed galvanotaxis, and are shown to be involved in wound healing, embryogenesis, and even cancer metastasis. Utilizing photolithography, we have created a chip-based platform capable of monitoring cellular galvanotaxis in one-, two-, and three-dimensional settings. We found that galvanotaxis is a complex tug-of-war between mechanisms in favor of cathodic versus anodic response. We have also identified the novel roles of Syndecan-1, a membrane-bound heparan sulfate proteoglycan, in sensing and directing glioblastoma cells to migrate toward the cathode by localizing at the anodal face. This is the first direct evidence supporting the electrophoresis-based galvanotaxis model and provides valuable insights into the underlying mechanism. The pathological implications of galvanotaxis on the invasiveness of glioblastoma cells are also discussed.
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Chapter 1 Overview

This dissertation focuses on the study of galvanotaxis, which is the directional migration of cells in the presence of a direct current electric field (dcEF). It is organized in the following way: Chapter 1 provides an overview of galvanotaxis including its hypothesis, physiological relevance, and implications; Chapter 2 describes the microfluidic platform designed for this study and provides the physical characterization observed using 3T3 fibroblast cells; Chapter 3 focuses on the biomolecular basis of directional sensing and the roles of endogenous brain electric fields on the invasion of glioblastoma cells; Chapter 4 summarizes our findings and provides directions for future experiments.

1.1 Introduction to galvanotaxis

1.1.1 Origin of endogenous electrical fields

The asymmetric distribution of ion channels and pumps between the apical and basal surfaces of the endothelial cells surrounding most organs leads to a transendothelial (or transepithelial) potential difference $\Delta \phi = \phi_{\text{apical}} - \phi_{\text{basal}}$ of +15 to +60 mV, corresponding to a dc electric field of 0.5 - 5 V cm$^{-1}$ [1-3]. This is a relatively small field, about six orders of magnitude lower than the threshold field for electroporation of a cell membrane (approx. 2 x 10$^6$ V cm$^{-1}$) [4]. However, epithelial and endothelial cells are programmed to sense and respond to dc electric fields (dcEFs) in processes such as wound healing, development, and nerve regeneration [1-3, 5-8]. Electric fields are also thought to play a role in angiogenesis [3] and metastasis [1].

1.1.2 Galvanotaxis in vitro

In cell culture, electric fields influence cell division, polarity, shape, and motility. Most cell types exhibit galvanotaxis where the majority of which, including fibroblast, leukocytes, stem
cells, keratinocytes, and neural cells respond to dcEFs by preferentially migrating to the cathode; however, a subset of cells including human granulocytes, human vascular endothelial cells, and metastatic human breast cancer cells were reported to migrate toward the anode [2] [9]. *In vitro* studies of galvanotaxis are usually performed in 2D by analyzing the path traced by individual cells in the presence or absence of an electric field. Most cell types respond to dcEFs of the magnitude of endogenous electric fields, however, the origin of this directionality and the mechanism of galvanotaxis remain unknown.

1.2 Galvanotaxis of 3T3 fibroblasts: a physical and morphological study

1.2.1 Physical characteristics of galvanotaxis in 2D

A marked characteristic of galvanotaxis in 2D is the perpendicular alignment of cells to the electric field during migration. Cooper et al., first observed that neural crest retracted both their anode- and cathode-facing margins and migrated laterally toward the cathode at a 5V cm$^{-1}$ field and suggested that the perpendicular alignments were resulted from modified ion fluxes [10]. However, the underlying mechanism is still largely unknown these days. Detailed analysis in many other cell types later revealed that cell motility, directional bias, and orientation can be positively influenced within a range of magnitudes of electric fields [11].

1.2.2 Galvanotaxis under physical confinements

For many processes of physiological interest cell motion is physically confined. For example, during migration through the extracellular matrix, cells migrate along channels between bundled collagen fibers [12]. Similarly, during wound healing, cells must squeeze between other cells. Various cell migration chambers have been developed for the study of galvanotaxis in 2D [13-
Our objective is to develop a versatile microfluidic-based platform to study galvanotaxis in 2D and confined geometry.

1.3 Galvanotaxis of brain tumor initiating cells (BTICs): an invasion and mechanistic study

1.3.1 Endogenous brain electric fields
Endogenous brain electric fields have traditionally considered to be an epiphenomena of structured neuronal network activity until recently discovered that brain electric fields could guide neocortical network activity [19]. Given the complexity in geometry and the inhomogeneous nature of brain tissue conductivity, the magnitudes of endogenous brain electric field are very hard to measure or estimate. One of the most accurate way of understanding endogenous electrical activity involves measuring local field potentials (LFP) using microelectrodes embedded within neuronal tissues [20]. In ferrets, the median peak endogenous field strength was measured around 2.29 mV/mm [19], whereas the maximal field was reported to be 3.5 mV/mm in adult mouse brains [21]. Yet, given the differences in size and the amount of activity, human brain endogenous brain electric fields may be significantly higher. In addition, the pathological effects of diseases such as Parkinson’s disease, Alzheimer's disease, and glioma, on endogenous brain electric fields is also largely unknown.

1.3.1 Progression of glioblastoma
Glioblastoma is among the most aggressive types of cancer with a median survival time only slightly more than a year. Malignant glioma cells tend to spread along blood vessels in the perivascular space or the white matter tracks within the brain parenchyma[22]. The diffusive nature of invasion often shelters tumor cells from surgery and radiation and thus imposes a great
challenge in the treatment of glioblastoma. An emerging strategy in treating glioblastoma focuses on a subpopulation of brain tumor initiating cells (BTICs) residing in the perivascular niche that are capable of self-renewal and differentiation. Understanding how various chemical and physical signaling regulate the functionality and invasion of BTICs can lead to better treatment strategies against glioblastoma.

Glioblastoma cells are known to respond to various migration cues. Chemokines such as bradykinin, EGF and PDGF were shown to induce directional migration via chemotaxis, whereas physical parameters such as interstitial flow and contact guidance can also mediate invasion of BTICs[23]. More recently, direct current electric fields (dcEFs) of 3 mV mm\(^{-1}\) were found to exist between subventricular zone and olfactory bulb and were suggested to direct the migration of neuroblasts along the rostral migration stream[21]. Considering BTICs exhibit similar migration characteristics as the precursor cells from which they are derived and aberrant electrical discharges (seizures) are often associated with the disease, we wonder whether endogenous electric fields may also influence the invasion of BTICs? And if it does, what might be the mechanisms?

### 1.3.2 Proposed mechanisms for galvanotaxis

Galvanotaxis is the directional migration of cells in the presence of a dcEF and has been implicated to involve in many physiological processes such as wound healing, embryogenesis, and cancer metastasis [9]. Numerous cell types of different origins were shown to exhibit galvanotaxis, where most cells responded by migrating toward the cathode. Although the exact mechanisms are still largely unknown, the proposed mechanisms for galvanotaxis were summarized nicely in a recent paper [24]. Possible mechanisms of galvanotaxis include the following: 1. Asymmetric ionic flow and differences in the opening probability of voltage-gated
ion channels due to hyperpolarization at the anode side and depolarization at the cathode side
drive cell migration. 2. Electro-osmotic flow created at the substrate or at the plasma membrane
can induce shear stress response or apply mechanical forces on stretch-activated membrane
components. 3. Gradients of membrane surface receptors created by electrophoretic force trigger
local activation and directional migration. Considering the voltage differences across a cell and
the magnitude of forces associated with electro-osmotic flow is relatively small and unlikely to
drive directional migration, Allen et al., suggested that electrophoresis of cellular membrane
components is the main driving force for galvanotaxis in keratinocytes [24].
Chapter 2  Galvanotaxis of 3T3 fibroblasts: a physical and morphological study

Most galvanotaxis study focused on studying the migration in 2D, yet as mentioned earlier, many physiological processes such as, wound healing and embryogenesis, occur under confined geometry. Therefore, to understand how electric field direct cell migration in both 2D and under confinements, we have designed a microfluidic platform capable of conducting long-term live cell galvanotaxis experiments in both confined and unconfined environments. NIH 3T3 mouse fibroblast was selected in the preliminary study due to its prevalence in the study of cell migration. Using the microfluidic platform in combination with NIH 3T3 fibroblasts, we have reported on quantitative characterization of the physical and morphological aspects of the motility of 3T3 cells under an electric field and physical confinement. Although galvanotaxis is usually associated with directional bias towards the cathode or anode, we show here that the influence of dcEFs on motility is much more complex. In 2D (no confinement) and in an electric field, cells orient perpendicular to the field vector and migrate preferentially toward the cathode. Unexpectedly we show that the electric field exerts forces on the cells both parallel and perpendicular to the field. These forces are in competition with each other and ultimately govern the trajectories of the cells in the presence of an electric field. At low field, the cells migrate preferentially towards the cathode, however, the perpendicular component of the individual segment vectors is larger than the parallel component. In a larger field, there is a significant increase in average velocity and the parallel component of the individual segment vectors is larger than the perpendicular component. These results suggest that there could be at least two independent signaling pathways that influence cell motility in an electric field. To further probe the effect of perpendicular alignment on directed migration induced by the electric field, 3T3
cells were seeded inside 20 µm channels to physically prevent cell orientation during galvanotaxis. We found that physical confinement results in an increase in cell velocity both in the absence and presence of an electric field compared to migration in 2D. This result could be relevant in understanding galvanotaxis in vivo.
Figure 2-1 Microfluidic platform.

(A) Schematic illustration of the galvanotaxis platform for the study of galvanotaxis in 2D and under physical confinement. (B) Photograph of the platform on a 35 x 50 mm glass slide. (C) Cross-sectional illustration of the galvanotaxis platform. (D) Fluorescence image of a device with ten 20 µm channels. Prior to seeding cells in the device, the internal surfaces of the channels are coated with fibronectin to mediate cell-substrate interaction. FITC-conjugated fibronectin (green). (E) Photograph of the device in a live-cell chamber. (F) Photograph of AgCl electrodes fabricated by chloridization of silver wires. Inset: cross section SEM image showing the 20 µm AgCl layer on the silver wires.
2.1 Materials and methods

2.1.1 Microfluidic platform

There are two key features of our platform (*Figure 2-1*). (1) Silver chloride electrodes are integrated into the platform without the need for external salt bridges and solution reservoirs. By integrating the electrodes into the microfluidic platform, our device is sufficiently small to fit in a live cell chamber (*Figure 2-2D* and *E*) so that experiments can be performed under controlled relative humidity, CO₂ partial pressure, and temperature. As described below, by using silver chloride electrodes, the only species involved in the electrochemical reactions at the electrode surface is the consumption or generation of chloride ions. In this way, we can avoid the parasitic reactions associated with noble metal electrodes such as gold or platinum. (2) By controlling the width w of the channels relative to the cell diameter d<sub>cell</sub> we can study cell migration under an electric field in 2D (w >> d<sub>cell</sub>) or in confined dimensions (w ≤ d<sub>cell</sub>). Such quasi-1D migration is analogous to migration along fibers in 3D gels, such as extracellular matrix [25, 26].

Confinement experiments were performed in 20 µm, 50 µm, and 100 µm wide channels. Here we compare cell migration in 2D (w x h, 1000 µm x 80 µm) and in 20 µm channels (w x h, 20 µm x 80 µm); experiments in 50 µm and 100 µm channels showed intermediate behavior and hence did not provide any additional insight. The details of platform fabrication are provided in Supporting Information.

2.1.2 Fabrication of Ag/AgCl electrodes

The electric field was applied between two Ag/AgCl electrodes located at each end of the microfluidic channel (*Figure 2-1*). Ag/AgCl electrodes have the advantage that the equilibrium at both electrodes is established by the reaction AgCl + e⁻ ↔ Ag + Cl⁻ and that the current is carried by chloride ion transport. The electrodes were fabricated by electrochemically forming
AgCl on two inch lengths of 0.025 inch diameter silver wire (A-M systems, Sequim, WA) using standard procedures [27]. Briefly, the silver wire and a platinum foil cathode were immersed in 1M HCl solution and chloridized for 30 minutes at a current of 5 - 10 mA cm$^{-2}$. The wires were then removed from the HCl solution, rinsed and stored in distilled water (Figure 2-1F). The electrodes were coiled to ensure that about two inches was embedded in the agarose gel (see Supporting Material for details). From scanning electron microscope images (Figure 2-1F), we determine an average AgCl thickness of 20 µm. A current of about 75 µA is required to maintain a field of about 5.5 V cm$^{-1}$ within a 1 cm long and 1000 µm wide channel (cross sectional area of 0.08 mm$^2$), and from Faraday’s law, we determine that a 5.5 V cm$^{-1}$ field can be maintained for more than six hours. Experiments were performed in the absence of a field, and with an electric field of 2.2 V cm$^{-1}$ or 5.5 V cm$^{-1}$. The magnitude of the field was confirmed using a four point probe method with two platinum wires inserted at each end of the channel.

2.1.3 Maintenance of cell lines

3T3 mouse fibroblast cells (ATCC) were cultured in DMEM media with 10 vol.% calf bovine serum (CBS) and 1 vol.% penicillin-streptomycin (PenStrep). Prior to experiments, cells were washed with phosphate buffer (PBS) and lifted from the surface using trypsin (0.25% trypsin-EDTA in PBS, Sigma). Suspended cells were centrifuged and re-suspended in 500 - 1000 µL of media before introducing into the platform through the cell injection port using a syringe. The cells were introduced at a density of 300,000 cells mL$^{-1}$.

2.1.4 Imaging

Time-lapse phase-contrast images were collected using an inverted microscope with a 10X objective (TiE2000, Nikon, Melville, NY). A live-cell chamber was used to control temperature (37 °C), CO$_2$ (5 vol.%), and relative humidity (95 %) (Figure 2-1E). The device was located in
a custom holder placed on an automatic stage in the live-cell chamber. The holder was customized with electrical feed-throughs to connect the electrodes to an external power supply (Figure 2-1E). Time-lapse images were taken at 5 min intervals for three hours using NIS-element software (Advanced Research Edition 3.22.11, Nikon, Melville, NY).

2.1.5 Image analysis

Time-lapse images were stacked using ImageJ (Version 1.45, NIH, Bethesda, MD) and loaded into Metamorph (Version 7.7, Molecular Devices, Sunnyvale, CA) for analysis of cell paths. Each cell path is made up of individual segments determined by the time between images (Δt = 5 min). Cell paths were only analyzed if the cells did not divide, were not in contact with other cells, and did not leave the field of view. The cell position in each frame was determined from the position of the nucleus. The cell path data was then analyzed using Matlab (MathWorks, Natick, MA). For each cell we determine the persistency, the cell velocity in each segment, the angular orientation of a segment with respect to the field vector, the average directedness, the segment turn angle, and the orientation of the cell with respect to the field vector (Figure 2-2E-G).

(1) The velocity v(t) is determined from the displacement Δzi and elapsed time between successive images (each segment along the cell path).

(2) The persistency (or directional persistence) p is a measure of the linearity of a path and is determined from r/∑Δzi where r is the overall displacement (distance between the start and end points) and ∑Δzi is the length of the path (i.e. the sum of the individual segments along the path). The persistency varies between 0 and 1: p = 0 corresponds to no displacement whereas p = 1 corresponds to a linear path (maximum bias). This term is also known as the directional
persistence and in studies of chemotaxis is known as the chemotactic index (CI) [28]. Note that
the terminology can be misleading since the persistency does not indicate whether a path is
aligned with an external bias, such as electric field or chemoattractant.

(3) The directedness $d_i$ is a measure of the directional bias of each segment and is given by $d_i = \cos(\phi_i)$, where $\phi_i$ is the angular orientation of a segment vector ($\overline{z_i}$) with respect to the field
vector and $0^\circ \leq \phi \leq 180^\circ$. $d_{av} = 0$ corresponds to random motion whereas values of ±1
correspond to linear motion parallel or antiparallel to the field vector. $d_{av} = 1$ ($\phi = 0^\circ$)
corresponds to the case where cells move in the direction parallel to the field vector, whereas for
$d_{av} = -1$ ($\phi = 180^\circ$) cell motion is antiparallel to the field. For all experiments in the presence of
an electric field, $d_{av}$ represents the average directedness over one hour during steady state motion
after the initial transient response.

(4) The segment turn angle $\delta_i$ is defined as the angle between two successive segment vectors, $\overline{z_i}$
and $\overline{z_{i+1}}$ (Figure 2-2F), and ranges between $0^\circ$ and $180^\circ$: $\delta = 0^\circ$ corresponds to no change in
direction whereas $\delta = 180^\circ$ corresponds to a complete reversal in direction. By analyzing the
distribution of segment turn angles, we can determine the correlation between each step under
the influence of a dcEF and under confinement.

(5) The cell orientation $\theta(t)$ is determined from the orientation of the long axis of the cell with
respect to the field axis as a function of time. $\theta(t)$ has values from $0^\circ$ - $90^\circ$ and the average value
is expected to be $45^\circ$ for a random distribution.
(6) Analysis of the mean square displacement (MSD) is often used to extract parameters such as the cell velocity and directional persistence. For comparison, details of data processing, analysis and results are provided in Supporting Material and Supplemental Figure S1.

2.2 Results and discussion

2.2.1 Galvanotaxis alters cell morphology and directs cell migration

Many cell types are known to respond to dcEFs with morphological changes and increased motility [10, 29, 30]. The exposure to dcEFs strongly dictates cell orientation and the direction of migration. In the absence of a dcEF, isolated 3T3 cells display typical fibroblast morphology with an elongated cell body and multiple protrusions (Figure 2A and Supplementary Video 1*) [31]. However, on application of an electric field, cells orient perpendicular to the field vector and are characterized by the formation of a broad lamellipodium extending the length of the cell and facing the cathode (Figure 2B and Supplementary Video 2*).

In the absence of an electric field, 53% of the cell paths (N = 43) were pointed in the direction of the field vector (towards the cathode) illustrating that there is no significant directional bias (Figure 2C). However, in the presence of an electric field, the cell paths are strongly biased in the direction of the cathode (Figure 2D) consistent with previous reports [30, 32]. In a 5.5 V cm\(^{-1}\) field, 92% of the cell paths (N = 38) were directed towards the cathode. The average velocity of 3T3 cells in the absence of a field was 0.38 ± 0.02 μm min\(^{-1}\) (SE) (Figure 2H). In a 2.2 V cm\(^{-1}\) field, there was no significant change in cell velocity (0.40 ± 0.03 μm min\(^{-1}\) (SE)), but cells preferentially moved toward the cathode and aligned in the direction perpendicular to the field. However, in a 5.5 V cm\(^{-1}\) field, the average velocity increased to 0.68 ± 0.03 μm min\(^{-1}\) (SE).
The persistency of the cells in the absence of a field was 0.60 ± 0.05 and remained approximately the same in a 2.2 V cm$^{-1}$ field (0.64 ± 0.02 (SE), P = 0.27) (Figure 2-I). Further increasing the field to 5.5 V cm$^{-1}$ significantly increased the persistency to 0.71 ± 0.05 (P < 0.01). The average directedness in the absence of a field was -0.02 ± 0.06 (SE). The directedness increased to 0.23 ± 0.03 (SE) in a 2.2 V cm$^{-1}$ field and 0.56 ± 0.12 (SE) in a 5.5 V cm$^{-1}$ field (Figure 2-J).

Despite the change in morphology and the increase in directedness of the cell path (Figure 2-J), the average cell velocity under a 2.2 V cm$^{-1}$ field (0.37 µm min$^{-1}$) is the same as experiments with no field (Figure 2-H). A larger field of 5.5 V cm$^{-1}$ results in an increase in the average cell velocity (Figure 2-H) and a further increase in the directedness (Figure 2-J). This result reveals a complex interplay between morphology, cell path, and cell speed, and suggests that cell velocity should not be used as the only parameter to probe the influence of electric field on cell motility.
Figure 2-2 Influence of electric field on the morphology of 3T3 cells and cell path.

(A) Phase contrast image of 3T3 cells in 2D with characteristic spindle shape and multiple filipodia. (B) Phase contrast image of 3T3 cells in a 5.5 V cm$^{-1}$ electric field showing perpendicular orientation with respect to the field, significant reduction in filipodia, and broad cathode-facing lamellipodia. (C) Trajectories of 3T3 cells in 2D (no field). Each cell path was analyzed and overlaid at the origin. With no bias, cells have equal probability to travel toward the left (black; 53%) and right (red; 47%) (N = 43). (D) In a 5.5 V cm$^{-1}$ field, the paths are strongly biased toward the cathode (92%, N = 38). (E) Schematic illustration of a cell path. (F) Cell paths are comprised
of individual segment vectors ($\mathbf{Z}_i$) characterized by the angle between the segment vector and the field vector ($\phi$), and the segment turn angle ($\delta$). (G) The cell orientation ($\theta$) is determined by the angle between the long axis and the field axis. (H) The average cell velocity does not change in a 2.2 V cm$^{-1}$ field ($P = 0.92$) but increases significantly in a 5.5 V cm$^{-1}$ field. (I) The average persistency of the cells also does not change in a 2.2 V cm$^{-1}$ electric field, but increases significantly in a 5.5V cm$^{-1}$ field. (J) The average directedness of the cells increases with the magnitude of the electric field. *, $P < 0.05$; **, $P < 0.01$; Student’s t-test. Data were obtained from three independent experiments, with 30 – 50 cells in each experiment. Error bars indicate standard error.
The average cell orientation was strongly influenced by the field. In the absence of a field, the average orientation was $44.8^\circ \pm 3.45^\circ$ (SE), whereas at 2.2 $\text{V cm}^{-1}$ and 5.5 $\text{V cm}^{-1}$, the average orientations were $71.5^\circ \pm 3.1^\circ$ and $80.3^\circ \pm 0.94^\circ$, respectively.

We next analyzed the velocity distributions for the individual segments in a cell path. The cell velocities follow an exponential distribution according to $f/f_0 = \exp(-(v-v_0)/\alpha_v)$, where $v_0$ is the most probable velocity (defined by $f_0$) and $\alpha_v$ is the characteristic velocity. Fits were determined for all velocities greater than or equal to the velocity at the peak of the distribution, $v_0$ (Figure 2-3A, D and G). In the absence of an electric field, $\alpha_v = 0.25 \mu\text{m min}^{-1}$ ($R^2 = 0.94$) (Figure 2-3A). Although the persistent random walk model for motility commonly used to describe individual cell motility predicts a Gaussian distribution [28, 33, 34], exponential velocity distributions have been reported for various cell types [35, 36], and it has been suggested that this behavior may be due to the limited production rate of ATP [35].

The distribution remains exponential with a similar exponent in a 2.2 $\text{V cm}^{-1}$ field ($R^2 = 0.99$) (Figure 2-3D), however, in a 5.5 $\text{V cm}^{-1}$ field, $\alpha_v$ increased to 0.62 $\mu\text{m min}^{-1}$ ($R^2 = 0.92$) (Figure 2-3G). This increase is consistent with the observation that the values of $v_0$ are around 0.20 $\mu\text{m min}^{-1}$ in each case and that the average velocity remains the same between 0 and a 2.2 $\text{V cm}^{-1}$ field but increases from 0.37 to 0.68 $\mu\text{m min}^{-1}$ between 0 and a 5.5 $\text{V cm}^{-1}$ field. The coefficient ($\alpha_v$) is proportional to the average cell speed (Fig. S2).

To characterize the orientation of individual segments, we analyzed the angular orientation of the segment vectors and the distributions of the parallel ($\Delta x$) and perpendicular ($\Delta y$) components of the displacements in each segment. In the absence of an electric field, the angular distribution of segment vectors $\phi_i$ is relatively uniform with an average value of $42^\circ$ (Figure 2-3B). Thus, the
corresponding scatter plot of the x- and y-components of each segment is symmetrical about the origin (Fig. S3A). The distributions of the parallel and perpendicular components of each segment overlap and are characterized by a gaussian distribution centered around zero (Figure 2-3B inset). In a 2.2 V cm\(^{-1}\) field, a peak around 70° indicates that cells move more in the direction perpendicular to the electric field (Figure 2-3E) than parallel to the field. The tendency is further shown in the corresponding scatter plot (Fig. S3B). In the presence of a 2.2 V cm\(^{-1}\) field, the distribution of \(\Delta y\) becomes broader and the frequency of \(\Delta x\) is significantly higher than \(\Delta y\) in the range of ± 2 µm (Figure 2-3E inset), indicating that the average perpendicular component of the displacement is greater than the parallel component. In a 5.5 V cm\(^{-1}\) field, the distribution of \(\phi_i\) becomes strongly biased to small angles with respect to the field vector and can be described by an exponential function according to \(p/p_0 = \exp(-\phi/\alpha_\phi)\), where \(p_0\) is the probability at \(\phi = 0^\circ\) and \(\alpha_\phi\) is the characteristic segment angle. From a fit we obtain a coefficient \(\alpha_\phi = 47.6^\circ\) in a 5.5 V cm\(^{-1}\) field (\(R^2 = 0.89\)) (Figure 2-3H), indicating that most segment vectors are within 48° of the field vector. A 5.5 V cm\(^{-1}\) electric field also significantly biased the distribution of \(\Delta x\) towards the cathode making it no longer symmetric around zero (Figure 2-3H inset).

The distribution of segment turn angles \(\delta_i\) can be described by an exponential function according to \(p/p_0 = \exp(-\delta/\alpha_\delta)\), where \(p_0\) is the probability at \(\delta = 0\) and \(\alpha_\delta\) is the characteristic turn angle. In the absence of a field (Figure 2-3C), \(\delta\) is weakly exponential with \(\alpha_\delta = 37.3^\circ\) (\(R^2 = 0.79\)). The exponent decreases progressively with increasing field with \(\alpha_\delta = 30.3^\circ\) (\(R^2 = 0.77\)) in a 2.2 V cm\(^{-1}\) field and \(\alpha_\delta = 21.5^\circ\) (\(R^2 = 0.79\)) in a 5.5 V cm\(^{-1}\) field (Figure 2-3F and I). The segment turn angle and cell velocity distributions remain exponential in the presence of an electric field indicating that the mechanism of motility is the same despite the dramatic change in cell
morphology (Figure 2-2A-B). Furthermore, there is no obvious influence of the dominant perpendicular contribution to cell motion seen at 2.2 V cm\(^{-1}\) (Figure 2-3E). It has been suggested that the exponential distribution of segment turn angles reported for the migration of eukaryotic cells is due to the random nucleation of an actin-protein complex anchored to the leading edge of a protrusion [37].

As shown above, the angular orientation (\(\phi\)) of individual segments is random in the absence of a field (Figs. 3B and S3A) but becomes biased in the presence of a field. In a 2.2 V cm\(^{-1}\) field, the distribution of \(\phi\) becomes biased with a broad peak between 60° and 90°, indicating that the perpendicular component of the bias is larger than the parallel component (Figs. 3E and S3B). Although the cell paths are biased towards the cathode, as seen by the increase in average directedness from 0 to 0.23 (Figure 2-2J), the cells are biased to move preferentially perpendicular to the field vector at small fields. Increasing the electric field to 5.5 V cm\(^{-1}\) further biased the trajectories toward the cathode, however, the parallel component of cell motion becomes dominant and the distribution of \(\phi\) becomes exponential (Figure 2-3H). The coefficient \(a_\phi = 47.6^\circ\) implies that most segment vectors are within about 45° of the field vector. This can be seen more clearly in the scatter plot (Fig. S3C). These results suggest a complex response to the electric field. There is a global bias towards the cathode, as well as a mechanism that results in motion perpendicular to the field vector at low fields. At high field, the perpendicular component is dominated by a horizontal component parallel to the field vector, resulting in an exponential distribution of segment angles mostly within a characteristic cone of about ± 45° around the field vector.
Figure 2-3 Distributions of average cell velocity, segment orientation (φ), and segment turn angle (δ) in 2D with no field, in a 2.2 V cm⁻¹ field and in a 5.5 V cm⁻¹ field.

The distribution of velocity is exponential both in the absence of an electric field ($R^2 = 0.94$) (A), in a 2.2 V cm⁻¹ ($R^2 = 0.99$) (D), and in a 5.5 V cm⁻¹ field ($R^2 = 0.94$) (G). In the absence of an electric field, φ is uniformly distributed (B) and the distribution of the horizontal displacement ($\Delta x$) and vertical displacement ($\Delta y$) of individual segments overlap (B, inset). In a 2.2 V cm⁻¹
field the perpendicular component of cell motion is larger than the horizontal component as shown by a peak around 70° (E), and thus, Δy has a greater effect on cell motion as indicated by the broader distribution of Δy around zero (E, inset). The distribution becomes exponential in a 5.5 V cm\(^{-1}\) field as the parallel component of the bias becomes dominant (H). The distribution of Δx also shows bias toward the negative-x (cathode) direction (H, inset). The distribution of δ is exponential under no field (C) and field (F and I). The solid lines are least squares fits to an equation of the form \(f/f_0 = \exp(-x/\alpha)\). (J) The average cell speed coefficient \(\alpha_v\) increases in a 5.5 V cm\(^{-1}\) field. (K) The average segment turn angle coefficient \(\alpha_δ\) is smaller in the presence of a 5.5 V cm\(^{-1}\) electric field but not significant. * P < 0.05; ** P < 0.01; Student’s t-test. Error bars indicate standard error. Statistics were obtained from at least two independent experiments with 30 - 50 cells in each experiment. Error bars indicate standard error.
In summary, in response to an electric field, 3T3 cells in 2D are biased towards the cathode. In a small electric field (2.2 V cm\(^{-1}\)), the average cell velocity and persistency are the same as with no field, however the cells reorient perpendicular to the electric field and are biased towards the cathode. Furthermore, the vertical component of each segment (perpendicular to the field vector) is larger than the horizontal component (parallel to the field). In a larger electric field (5.5 V cm\(^{-1}\)), the average velocity and directedness increase significantly. In addition, the horizontal component of the segments along a path become larger than the vertical component, and the segment vectors are generally within a cone of about ± 45° from the field vector. The distributions of cell velocities and segment turn angles are exponential in all cases. These results suggest that the mechanism of motility is due to a random process within the cell. However, the electric field modulates two processes, one that regulates cell motion perpendicular to the field and one that regulates cell motion parallel to the field. Globally, cell motion is not completely random in that a cell has limited ability to make large directional changes due to the orientation of protrusions associated with the spindle like morphology in the absence of a field and the broad cathode-facing lamellipodia in the presence of an electric field.

**2.2.2 Galvanotaxis is enhanced under confinement**

To further study the effects of perpendicular alignment in 2D galvanotaxis on motility, cells are confined within microchannels to prohibit the orientation in the perpendicular direction. In the presence of an electric field and without confinement, cells oriented perpendicular to the field vector with a length of around 100 µm (*Figure 2-2B*), therefore 20 µm wide channels aligned parallel to the field vector prevent the reorientation seen on an unconfined surface. In addition, spatial confinement allows us to impose quasi-1D motion on the cell paths. When confined to 20 µm channels, in the absence of an electric field, 53% of the cells (N = 40) moved in the direction
of the field vector although the cell paths were strongly oriented due to the spatial confinement (Figure 2-4B and Supplementary Video 3*). In the absence of a field, the average velocity in the channels was 0.68 ± 0.03 µm min⁻¹ (SE), significantly faster than cells with no confinement (P < 0.01) (Figure 2-4D).

Under confinement, cells were forced to align in the direction parallel to the channels. In most cases, cells migrated with the membrane in contact with the channel bottom and the channel walls. In the presence of a field, instead of a broad and persistent cathode-facing lamellipodia, as seen under no confinement (Figure 2-2B), cells tended to contract their trailing edge and migrated with a smaller projected area and with the cell body becoming less flat (Figure 2-4A and Supplementary Video 4*). In the presence of a 5.5 V cm⁻¹ field, 100% of the cells (N = 35) moved in the direction of the field vector. However, as we discuss later, this does not mean that all segments along a path were in the direction of the field vector.

In the presence of a field, cells migrated rapidly in the direction of the cathode (Figure 2-4C). For all experiments in 20 µm channels, the average velocity was significantly larger than the corresponding case with no confinement (P < 0.01) (Figure 2-4D). In a 2.2 V cm⁻¹ field, the average velocity was 0.70 ± 0.02 µm min⁻¹ (SE), similar to the velocity in the absence of a field (P = 0.45). For a 5.5 V cm⁻¹ field, the average velocity was 1.02 ± 0.07 µm min⁻¹ (SE), significantly larger than for a 2.2 V cm⁻¹ field (P < 0.01). The increase in cell velocity in the channels can be due to either the change in cell morphology dictated by the spatial confinement or by the influence of channel walls on motility. An increase in cell speed has also been reported for 3T3 cells on quasi-1D lines modified with fibronectin formed by photopatterning and was thought to be due to the suppression of cell spreading and lateral lamellae [38].
The persistency in 20 μm channels was significantly higher than the corresponding case with no confinement (P < 0.01). However, the persistency under confinement did not change significantly with electric field. In the absence of a field, the persistency in the channels was 0.71 ± 0.03, whereas in a 2.2 V cm⁻¹ field the persistency was 0.78 ± 0.03 (P = 0.08). Further increasing the field to 5.5 V cm⁻¹ had no obvious effect on persistency (P = 0.36) (Figure 2-4E).

In the absence of an electric field, the average directedness in the channels was 0.01 ± 0.1 (SE), very similar to cell motion without confinement (Figure 2-4F). The average directedness increased to 0.48 ± 0.06 (SE) under a 2.2 V cm⁻¹ field, significantly higher than experiments without confinement (Figure 2-4F). Under a 5.5 V cm⁻¹ field, the directedness increased to 0.71 ± 0.04 (SE), very similar to experiments without confinement under the same field (P = 0.29), but significantly higher than for a 2.2 V cm⁻¹ field (P < 0.05). These results indicate that the influence of confinement is inversely related to the magnitude of the field. In a low field, confinement induces significant directional bias, whereas in a high field the directional bias is dominated by the field.

The velocity distributions remain exponential for motility in 20 μm channels both in the absence and presence of an electric field, indicating that the mechanism governing cell speed is the same in all cases, despite the differences in cell morphology. It has been suggested that cell migration under confinement is less dependent on integrin-mediated adhesion but depends largely on microtubule dynamics [39, 40] and actin polymerization at the cell membrane [41]. Therefore, although physical confinement can induce cytoskeletal alterations, the rate-limiting step that governs cell speed in unconfined and confined geometry may be the limited production rate of ATP, which gives rise to the exponential distribution of cell speed [35]. The distributions were fit using the same procedure as described previously. The coefficient v₀ was independent of the
field, with values of 0.13 μm min\(^{-1}\) (no field), 0.13 μm min\(^{-1}\) (2.2 V cm\(^{-1}\)) and 0.14 μm min\(^{-1}\) (5.5 V cm\(^{-1}\)) (Figs. S4A, D, G). The exponents increased with increasing field, with \(\alpha_v = 0.5 \text{ μm min}^{-1}\) \((R^2 = 0.88; \text{no field})\), \(\alpha_v = 0.66 \text{ μm min}^{-1}\) \((R^2 = 0.89; 2.2 \text{ V cm}^{-1})\), and \(\alpha_v = 1.2 \text{ μm min}^{-1}\) \((R^2 = 0.92; 5.5 \text{ V cm}^{-1})\) (Figs. S4A, D, G), however, the coefficients are comparable to the values obtained with no confinement (Fig. S4J).

The influence of confinement on cell trajectory was further characterized by examining the angular orientation of the segment vectors and the segment turn angle (Fig. S4). In the absence of a field, the segment orientation angle \(\phi\) with respect to the field vector was bipolar and symmetrical (Fig. S4B) since cells have equal probability to travel to the left or the right in the channels. The distribution around zero is exponential with \(\alpha_\phi = 5.9^\circ\). This is in contrast to cell motion on a 2D surface where the distribution of angles is flat (Figure 2-3B). In the presence of a 2.2 V cm\(^{-1}\) field, the distribution becomes asymmetric due to the bias on the cell paths as shown by the higher probability of \(\phi\) near zero (Fig. S4E). The distribution around zero remains exponential with \(\alpha_\phi = 5.1^\circ\). In a 5.5 V cm\(^{-1}\) field, the probability of \(\phi\) near zero further increases and can be characterized by an exponential distribution with \(\alpha_\phi = 5.2^\circ\) (Fig. S4H). However, even though 100% of the cells moved toward the cathode in the presence of a 5.5 V cm\(^{-1}\) field, a small subset of segments along a path were pointed in the direction against the field vector as shown by the small peak near \(\phi = 180^\circ\).

The segment turn angle, in the absence of a field, is bipolar but asymmetric with low probability around 180\(^\circ\) (Fig. S4C). The distribution around zero is exponential with \(\alpha_\delta = 7.7^\circ\). In the presence of a 2.2 V cm\(^{-1}\) field, the distribution becomes more polarized (Fig. S4F) but the distribution around zero remains exponential with \(\alpha_\delta = 6.2^\circ\). Further increasing the field to 5.5 V cm\(^{-1}\) has no significant effect on the distribution of \(\alpha_\delta\) (Fig. S4I) around zero, which remains
exponential with $\alpha_s = 7.0^\circ$. The segment turn angle coefficients obtained in 20 $\mu$m channels are significantly smaller than the values obtained on 2D surfaces (Fig. S4K).
Figure 2-4 Confinement in 20 μm channels influences galvanotaxis.

(A) A series of phase contrast images taken at 30 min intervals showing motion of a cell in a 5.5 V cm\(^{-1}\) field. Cell paths with no field (B) and in a 5.5 V cm\(^{-1}\) field (C). Note that the axes are not isotropic. In the absence of a field, 53\% of the cells moved towards the cathode, whereas in a 5.5 V cm\(^{-1}\) field, 100\% of the cells moved towards the cathode. (D) In the absence of a field, the average velocity increased under confinement (v = 0.68 μm min\(^{-1}\) ± 0.03 S.E.) compared to no confinement (v = 0.37 μm min\(^{-1}\) ± 0.04 S.E.). In a 2.2 V cm\(^{-1}\) field, there was no change in average cell velocity (P = 0.45). A 5.5 V cm\(^{-1}\) field resulted in an increase in average velocity (P < 0.01). Black bar: 2D, Gray bar: 20μm channel (E) Spatial confinement significantly increases the average persistency of the cell paths comparing to no confinement. The influence of electric
field on cell persistency is not significant. (F) In the absence of a field, the directedness remained close to zero under confinement. The directedness under confinement increased with increasing field. ** P < 0.01. Student’s t-test.
2.2.3 Transient response in directedness and orientation

In addition to the steady state behavior, we also studied the transient response to an electric field. The key parameters in the transient response are the directedness and the overall cell orientation. In the absence of confinement, on application of a 5.5 V cm\(^{-1}\) electric field, cell re-orientation occurs over about 2 hours (Figure 2-5A). The time-lapse images show that cells first send out protrusions in the desired locations to align perpendicular to the field vector before developing stable cathode-facing lamellipodia. The time dependence of the average directedness of the cells follows an exponential increase (Figure 2-5B) with a time constant \(\tau\) of 43.5 minutes (Figure 2-5D). Similarly, the average cell orientation angle (\(\theta_{\text{avg}}\)) increases exponentially (Figure 2-5C) with a time constant \(\tau\) of 40 minutes (Figure 2-5D). The progressively smaller error bars indicate that cell orientation gradually converges to a steady state perpendicular to the field vector. Cell orientation also increased exponentially in the presence of a 2.2 V cm\(^{-1}\) field (Fig. S5); however, cells aligned substantially slower as suggested by a greater time constant (\(\tau = 95\) minutes).
Figure 2-5 Response to an electric field in 2D.

(A) Time-lapse images showing the reorientation of a 3T3 cell in a 5.5 V cm\(^{-1}\) electric field. The time (min) after applying the electric field is indicated in the images. (B) The average directedness (d) of 3T3 cells versus time after applying a 5.5 V cm\(^{-1}\) field. (C) The average cell orientation (θ) versus time after applying a 5.5 V cm\(^{-1}\) field (N = 36). The solid lines represent fits to an equation of the form \((x - x_{\text{min}})/(x_{\text{max}} - x_{\text{min}}) = 1 - \exp(-t/\tau)\) where \(x = d\) or \(θ\) and \(τ\) is the time constant. For the directedness, \(d_{\text{min}} = 0\) and \(d_{\text{max}}\) is the average directedness from 120 - 180 min. (D) Time constants for the transient change in directedness and cell orientation after applying a 5.5 V cm\(^{-1}\) field.
2.3 Conclusions

Through detailed analysis of cell paths we show that the influence of electric field on motility is much more complex than simply imposing a directional bias towards the cathode or anode. External electric fields with magnitude similar to endogenous electric field modulate several processes within 3T3 cells, involving overall cell reorientation, and motion both parallel and perpendicular to the field. Detailed analysis of cell paths reveal forces both parallel and perpendicular to the electric field that drives migration in a field dependent manner. Confinement in 2D channel prevents cell orientation perpendicular to the field and results in increased cell velocity. These results provide new insight into the biophysical response of cells to electric fields that can guide further research into the signaling pathways that regulate galvanotaxis.
2.4 Supporting information

2.4.1 Fabrication of microfluidic platform

The galvanotaxis platform includes a central microfluidic channel or channels located between two media reservoirs and integrated electrode ports. A separate cell injection port is located at one end of the microfluidic channel. To fabricate the mold for the device, an 80 µm thick photoresist film (SU-8 2075, Microchem, Newton, MA) was first spin coated on a silicon wafer. The photoresist was then exposed in a mask aligner (OAI, San Jose, CA) using a custom-designed mask. Next, a second 80 µm thick photoresist layer was spin coated onto the first layer and after alignment was exposed so that the mold was 160 µm thick everywhere but 80 µm thick in the main channel. Next, the unexposed photoresist was removed using SU-8 developer (Microchem, Newton, MA).

Devices were fabricated by pouring polydimethysiloxane (PDMS) (Dow Corning, Midland, MI) on the mold and curing at 65 °C. Subsequently, nylon tubes (part 91145A161, McMaster, Robbinsville, NJ) that served as reservoirs, were carefully located at the desired locations before the second layer of PDMS was cast and cured over the mold.

The inlets and outlets, located in the center of each reservoir, were formed using a 2.5 mm punch (Harris Uni-core, Ted Pella, Redding, CA). The inlet of the cell injection port was created by punching through the PDMS with a 30-gauge blunt head needle (Fisnar, Wayne, NJ). Each PDMS device was bonded to a No. 1 coverslip (Fisher Scientific, Pittsburgh, PA) by exposing to an oxygen plasma for 50 seconds (Plasma Etch, Carson, Nevada). All experiments reported here were performed in a 1000 µm channel (no confinement, 2D) or a 20 µm channel.
2.1.2 Platform integration

Immediately after assembly, the microfluidic platform was placed in a sterile tissue culture hood. The main channel was incubated with 200 µL of 50 µg mL⁻¹ FITC conjugated fibronectin (BD) solution for one hour. The device was then washed with phosphate buffer (PBS, Gibco) and the reservoirs and channel filled with cell media.

The Ag/AgCl electrodes were integrated directly into the device by embedding in agarose gel. This approach allows us to avoid conventional salt bridges and external solution reservoirs containing the electrodes. 2 wt.% of cell-culture grade agarose powder (Invitrogen) was dissolved in Steinberg’s solution by gently heating the solution at 130 °C. Prior to adding the agarose solution into the electrode reservoirs, the media reservoirs were capped with rubber septa to create a positive pressure that prevented agarose from flowing into the channels during gelation. 500 µL of the agarose solution was added to each electrode reservoir. An Ag/AgCl electrode was then immediately inserted into the agarose solution and the electrode port was sealed with a centrifugal filter (Millipore, Billerica, MA) to ensure a good contact between the electrode and agarose and to prevent evaporation. The devices were then left in a tissue culture hood for 30 minutes to ensure the agarose had completely gelled. A constant voltage was applied by connecting two Ag/AgCl electrodes embedded in agarose to a potentiostat (VersaStat 3, Princeton Applied Research). The actual voltage drop across both ends of the channel was measured using a four-point probe method with two platinum wires. The electric field was further verified based on the resistivity of the media inside the channel and the current recorded by the potentiostat.
2.1.3 Analysis of mean square displacement

The mean square displacements (MSD) for individual cells were determined from the experimentally acquired cell paths using overlapping intervals [42]. The persistent random walk (PRW) model is commonly used to analyze cell motility in 2D [28, 33, 34]. This model is described by:

$$\langle d^2 \rangle = 2S^2P\left( t - P\left( 1 - \exp\left( -\frac{t}{P} \right) \right) \right)$$

where $\langle d^2 \rangle$ is the mean square displacement (MSD), $S$ is the root-mean square speed, and $P$ is the directional persistence time [33]. Several modifications to further improve the applicability and consistency of the model have been proposed, however, there is no standard way of analyzing MSDs [36]. The MSD of all cells can first be averaged and then fit to the PRW model or first be fit to the PRW model and then averaged later. Here we present the results based on four common variations for applying the PRW model: unweighted average then fit (U/A), unweighted fit then average (U/F), weighted average then fit (W/A), and weighted fit then average (W/F). The weighting was used to account for the increasingly lower numbers of points at longer time intervals [37, 42]. In other words, the weighted model puts more emphasis on the initial points (smaller time interval) as shown in Figure S1B.

The results of the analysis of the cell paths using the PRW model are summarized in Figure S1. For cell motility in 2D in the absence of a field, the values of $S$ obtained from the MSDs were slightly larger than the values obtained from analysis of the individual segments, but were all within 20% with the exception of the U/F method. The best agreement between the average cell velocity obtained from the individual segments and the MSDs was obtained by calculating the
unweighted average of all the MSDs and then fitting to equation (1). In the absence of confinement and electric field, the average cell velocity obtained from analysis of the individual segments was 0.37 µm min\(^{-1}\) (Figure 2-3F) and from the MSDs was 0.44 µm min\(^{-1}\) (Fig. S1D, U/A). The cell velocity obtained from analysis of the individual segments was obtained for \(\Delta t = 5\) minutes and was weakly dependent on sampling time, as has been reported previously [43]. Extrapolation of the average cell velocity to \(\Delta t = 0\) (Fig. S1C) gives the instantaneous velocity of 0.44 µm min\(^{-1}\), in excellent agreement with the value obtained from analysis of the MSDs.

In the presence of an electric field, cell velocities obtained from MSDs were on average 30% higher than the value obtained from the individual segments with the U/A method giving the best agreement (18%). The difference further decreased when comparing to the instantaneous velocity.

Interestingly, in 20 µm channels, the cell velocities obtained from MSDs had better agreement with values obtained from analysis of individual segments. The average differences were 14% (no field) and 17% (5.5 V cm\(^{-1}\) field), respectively. When compared to the instantaneous velocity, the difference further decreased to 6% and 10%, respectively. Note that, the U/A method still yields the best result among the four methods tested.

In addition to the average cell speed \(S\), the directional persistence time \(P\) can also be extracted from analysis of the MSDs. Values for \(P\) obtained from fits to equation (1) (Fig. S1) varied between 30 - 80 minutes. In general, smaller values for \(P\) were obtained using weighted individual (W/F) or averaged (W/A). Comparison of the results shows no obvious influence of electric field or confinement. Similar results were obtained by arbitrarily defining the persistence as the time of migration where \(\delta < 70^\circ\) (Fig. S1).
Figure S1 Test Analysis of cell trajectories base on mean square displacement (MSD).

(A) Overlay of the MSD for each cell in a single experiment. (B) Effect of unweighted (UW) and weighted (W) persistent random walk (PRW) on the fit. The weighted PRW method put more emphasis on the initial points (smaller time intervals). (C) Instantaneous velocities (V-inst) are extrapolated from the average velocity calculated from the trajectories (V-traj) to Δt = 0. (D) Comparison of the average velocity (V-traj) and instantaneous velocity (V-inst) to root-mean square speed calculated from different methods under no confinement. U/A: unweighted average then fit, U/F: unweighted fit then average, W/A: weighted average then fit, W/F: weighted fit then average (W/F). (E) Comparison of V-traj and V-inst to root-mean square speeds in 20μm.
channel. (F) Comparison of persistence time (P) base on arbitrarily defining the persistence as time of migration where $\delta > 70^\circ$ and four variations of PRW model mentioned above.

![Graph showing $\alpha_v$ versus average velocity in 2D (open symbols) and in 20 μm channels (solid symbols). The solid line indicates that $\alpha_v$ is proportional to the average velocity. (□) control, (o) 2.2 V cm$^{-1}$, (Δ) 5.5 V cm$^{-1}$.]

**Figure S2.** $\alpha_v$ versus average velocity in 2D (open symbols) and in 20 μm channels (solid symbols). The solid line indicates that $\alpha_v$ is proportional to the average velocity. (□) control, (o) 2.2 V cm$^{-1}$, (Δ) 5.5 V cm$^{-1}$.

![Scatter plots of the x- and y-components of each segment in no field (A), a 2.2 V cm$^{-1}$ field (B), and a 5.5 V cm$^{-1}$ field (C). In the absence of a field, the scatter plot is symmetrical about the origin (A). In a 2.2 V cm$^{-1}$ field, $\Delta y$ has a greater effect on cell motion and therefore, the x- and y-components of each segment are scattered along the y-axis with a higher frequency between $60^\circ$ - $90^\circ$ and $-60^\circ$ to $-90^\circ$ (B). In a 5.5 V cm$^{-1}$ field, horizontal bias toward the cathode becomes dominant and the majority of the x- and y-components are within $\pm45^\circ$ (C).]

**Figure S3.** Scatter plots of the x- and y-components of each segment in no field (A), a 2.2 V cm$^{-1}$ field (B), and a 5.5 V cm$^{-1}$ field (C). In the absence of a field, the scatter plot is symmetrical about the origin (A). In a 2.2 V cm$^{-1}$ field, $\Delta y$ has a greater effect on cell motion and therefore, the x- and y-components of each segment are scattered along the y-axis with a higher frequency between $60^\circ$ - $90^\circ$ and $-60^\circ$ to $-90^\circ$ (B). In a 5.5 V cm$^{-1}$ field, horizontal bias toward the cathode becomes dominant and the majority of the x- and y-components are within $\pm45^\circ$ (C).
Figure S4. Distributions of average cell velocity, segment orientation (ϕ), and segment turn angle (δ) in 20 µm channels with no field (A, B, C), in a 2.2 V cm⁻¹ field (D, E, F), and in a 5.5 V cm⁻¹ field (G, H, I). The distribution of velocity is exponential in the absence of an electric field (R² = 0.88) (A), in a 2.2 V cm⁻¹ (R² = 0.89) (D), and in a 5.5 V cm⁻¹ field (R² = 0.92) (G). (B) In the absence of a field, the distribution of segment turn angles is bipolar and symmetrical with peaks at δ = 0° and 180°. (C) In the presence of a field, the approximately symmetrical distribution of δ becomes strongly
biased towards small angles. (F) There is relatively little change in the distribution of segment turn angles in the presence of a field. (G) Further increasing the electric field to 5.5 V cm⁻¹, significantly increases the strength of the exponential, α increases from 0.5 to 1.2 µm min⁻¹. (H and I) No further changes in the distribution of segment orientation and segment turn angle were seen in the presence of a 5.5 V cm⁻¹ field comparing to no field. (J) Comparison of α, between no confinement (black) and confinement (blue). (K) Confinement greatly decreases α. Similar to the results in 2D, electric field has no obvious effect on α under confinement.
Chapter 3  Galvanotaxis of brain tumor initiating cells (BTICs): an invasion and mechanistic study

To understand whether dcEF is a potent migration cue for the invasion of glioblastoma and whether the driving mechanism is different from other cell types, a chip-based galvanotaxis device capable of long-term observation was constructed using photolithography (Figure 3-1). The small form factor of the device eliminates the need of CO₂ independent media and allowed BTICs to maintain their stemness using stem cell media. Through both two-dimensional and three-dimensional migration study, we discovered that the migration of BTICs is sensitive to electric fields. A dcEF as low as 0.5 V cm⁻¹ was sufficient to significantly bias cell migration. Furthermore, the direction of galvanotaxis in 2D is closely regulated by the electrostatic interaction between the cells and the substrate independent of the type of extracellular matrices. Cells migrated toward the anode when seeded on a positively charged surface coated with poly-L-ornithine (PLO), whereas removal of the positively charged polymer layer surprisingly resulted in a reversal in directional response toward the cathode. More interestingly, we showed the cathodic response on neutral surfaces is highly regulated by membrane bound heparan sulfates. Heparan sulfates are highly localized toward the anodal face of the cells in the presence of an electric field due to the strong electrophoretic interaction. Enzymatic removal of heparan sulfate strikingly reversed the cathodic response observed on neutral surface back to anodic response. The reversal in directional response is further enhanced when both heparan sulfate and chondroitin sulfate were enzymatically removed. Immunostaining of heparan sulfate and Syndecan-1 showed a remarkable co-localization pattern indicating Syndecan-1 as a novel electric field sensor for cathodic response. We showed here for the first time an extracellular
membrane component capable of sensing and leading to the cathodic directional migration in the presence of an electric field and proposed an electrophoretic model for galvanotaxis.

Together, we conclude that galvanotaxis is indeed a tug-of-war between competing mechanisms as described previously [44]: a cathodic response regulated by the localization of heparan sulfate/Syndecan-1 and an anodic response with mechanisms that are yet to be identified. The delicate balance between the anodic and cathodic response is further regulated by the electrostatic interaction between cells and the substrate. Chemical inhibition assays in both 2D and 3D were further carried out to screen for additional molecular targets responsible for the directional response. Inhibiting PI3K in 2D partially attenuated the directional response; however, surprisingly, had little effects on cells embedded in a 3D ECM. Our results implicated the influence of endogenous electric fields on the invasion of glioblastoma cells and also highlighted the complexity of directional sensing involved in galvanotaxis. Both chemical and physical cues can interact with one and another and collectively determine the direction of cell migration. Physical constrains encountered in 3D migration can further influence cell migration mode and resulted in outcomes different from experiments in 2D.

3.1 Materials and Methods

3.1.1 Equipment and reagents

An inverted microscope (Nikon, Eclipse TiE) equipped with a confocal laser module and a live-cell chamber was used for all microscopy. Electric fields were applied via a potentialstat (Princeton Applied Research, VersaStat 3, Oak Ridge, TN) operating in a constant voltage mode while monitoring the current supplied. BTIC medium was made of DMEM/F12 (Invitrogen, US) supplemented with B-27 supplements (Invitrogen, US), hEGF (20 ng mL⁻¹, Peprotech, Rocky Hill, NJ) and hFGF (20 ng mL⁻¹, Peprotech, Rocky Hill, NJ). Poly-L-ornithine and laminin were
purchased from Sigma Aldrich (St. Louis, MO). Type I rat tail collagen was from Corning (Tewksbury, MA) and hyaluronic acid (Hystem) was from Sigma Aldrich. Latrunculin A, nocodazole, and LY294002 were from Sigma, whereas the rest of the chemical inhibitors were purchased from Tocris bioscience. Primary antibodies for α-tubulin (Abcam), phalloidin, and DAPI (Life Technology) were used at the concentration recommended by the manufacturer for immunofluorescence.

3.1.2 Cell lines

Early passage of human brain tumor initiating cells (BTICs), GBM 612, were used and previously validated by Johns Hopkins Genetic Resources Core Facility. GBM612 cells isolated from intraoperative tissue are multipotent and are able to form diffuse tumors when implanted into animal models [45]. BTICs were grown in culture flasks coated with poly-L-ornithine and laminin (Sigma, 1μg/mL) and cultured with stem cell media composed of DMEM/F12, B27 supplements, EGF, and FGF[46]. The molecular subtype of 612 is proneural as characterized previously using a metagene score based approach.

3.1.3 Two-dimensional galvanotaxis and cell tracking

Galvanotaxis of BTICs were carried out using a customized galvanotaxis chip reported previously utilizing standard microfabrication techniques (Figure 3-1A) [47]. Briefly, BTICs were seeded in a galvanotaxis chip coated with ECM of choice for 24 hours before mounted onto a microscope equipped with a live-cell chamber for time lapse experiments. In each experiment, electric fields were applied through a pair of Ag/AgCl electrodes embedded in agarose via a potentialstat operated in a constant-voltage mode while making sure the current was stable. Cells were stimulated in a dcEF for 3~9 hours before being fixed for immunofluorescence studies.
Time lapses of the experiments were subsequently analyzed where the trajectories of cells were automatically tracked using Metamorph (Molecular Devices, US) to minimize any tracking biases. Only isolated cells that remained in the field of view and did not undergo mitosis were selected to track. Cell trajectories were further analyzed using a customized Matlab (MathWorks, US) script to characterize physical parameters including cell motility and directedness. Here we defined cell motility as the total path length traveled by a cell divided by the duration of time and directedness is defined as \( \sum_{i=1}^{n} \cos \theta_i / n \), where \( n \) is the total number of cells and \( \theta_i \) is the angle between the vector of cell displacement and EF vector.

### 3.1.4 Drug inhibition assay

For each drug inhibition study, cells were treated with the compounds at the indicated concentration for at least three hours before experiments. The name and the concentration of each compound used are the following: Latrunculin A (250 ng mL\(^{-1}\)), nocodazole (200ng mL\(^{-1}\)), ZCL278 (50 \( \mu \)M), NSC23766 (50 \( \mu \)M), Y27632 (50 \( \mu \)M), LY294002 (50 \( \mu \)M), PD158780 (0.5 \( \mu \)M), Imantitib (1 \( \mu \)M), AMD3100 (50 \( \mu \)M), and SB225002 (1 \( \mu \)M).

### 3.1.5 Immunofluorescence study

BTICs were fixed with 3.7% formaldehyde, permeabilized or non-permeabilized based on the antibody, and stained following standard procedures. Primary antibodies were used at a 1:100 dilution and incubated overnight at 4\(^{\circ}\)C. Secondary antibodies conjugated to fluorophores were used at 1:200 dilution and incubated for an hour before washing and imaging. Immunostaining of neurospheres for stem cell markers were carried out following the procedures reported previously [48].
3.1.6 Quantitative analysis of localization

Cells stained with heparan sulfate were imaged using a confocal microscope at 40X. For each cell, a region of interest (ROI) around the cell border was first identified using ImageJ (version 1.46r) and then divided into two halves, the cathode facing side \((C)\) and anode facing side \((A)\), vertically along the cell’s geometric centroid (Figure 3-4C). To evaluate localization, a Wilcoxon signed-rank test under the presumption where \(A\) is greater than \(C\) was performed among the top 10% pixel intensities within \(A\) and \(C\). A p-value < 0.05 is defined as a localization toward the anode here.

3.1.7 Galvanotaxis of BTICs embedded in a three-dimensional matrix

BTICs were embedded in a three-dimensional matrix composed of a mixture of type I collagen and hyaluronic acid and stimulated with a dcEF to provide a more physiological relevant condition. Briefly, tumor cells \((10^5)\) were seeded in 100 \(\mu\)L of gel made of 1 mg mL\(^{-1}\) of type I collagen and 1 mg mL\(^{-1}\) of hyaluronic acid and injected into the galvanotaxis chip via the cell injection port (Figure 3-1B). The gel composition was previously optimized to recapitulate glioma invasion in vitro and had a comparable stiffness to the brain ECM [23]. The cell/ECM mixture was allowed to polymerize for half an hour at 37 °C before 50 \(\mu\)L of medium was added into both media reservoirs to keep the gel hydrated. The final dimension of the gel was defined by the channel size and measured to be 1 cm x 5 mm x 250 \(\mu\)m (LxWxH). Cells were allowed to spread for at least 36 hours before mounting onto a microscope for time-lapse experiments. Only cells embedded in the center of the gel and stayed within the same focal plane throughout the designated time frame were tracked and analyzed to represent galvanotaxis in a three-dimensional matrix.
Figure 3-1 A chip-based galvanotaxis device capable of studying two- and three-dimensional galvanotaxis.

(A) Photo of a galvanotaxis chip bonded on a 35mm x 50 mm coverslip. Each chip contains two sets of symmetry designs for higher throughput. (B) Schematic illustration of the device. Each design contains two coiled Ag/AgCl electrodes embedded in agarose reservoirs and two media reservoirs separated by a cell culture channel in the middle. Cells alone or cells embedded in an ECM are introduced via the cell injection port and allowed to fully adhere before experiments. The actual dimension of the cell culture channel is 10 mm x 5 mm x 250 μm (LxWxH).
3.2 Galvanotaxis of BTICs

3.2.1 BTICs cultured on poly-L-ornithine/laminin surfaces migrated anodically in a voltage dependent manner

In the absence of a dcEF, 612 cells migrated in a non-directed manner with random orientation (Figure 3-2E). Cell motility in the absence of a dcEF was $0.31 \pm 0.04 \, \mu m \, min^{-1}$ and mean directedness was $-0.01 \pm 0.08$. (Figure 3-2G and H). In the presence of a weak electric field (0.5 V cm$^{-1}$), cells migrated slightly faster at $0.38 \pm 0.05 \, \mu m \, min^{-1}$ ($p=0.02$) (Fig 2G) but cell trajectories were significantly biased toward the anode and resulted in a directedness of $-0.40 \pm 0.06$ (Fig 2H). Further increasing the field to 1 V cm$^{-1}$ did not further enhance cell motility ($0.41 \pm 0.07 \, \mu m \, min^{-1}$), however, cell trajectories were significantly more biased toward the anode with a mean directedness of $-0.47 \pm 0.12$ (Figure 3-2H, Supplemental Video 1*). In response to the dcEF, cells also oriented with its long axis parallel to the direction of electric field and extended prolonged protrusions toward the anode (Figure 3-2D). Immunostaining of microtubules and actin filaments indicated the abundance of microtubules at the protrusions (Figure 3-2D) and confirmed the importance of microtubules in the migration of BTICs. Taken together, we have shown that dcEFs as low as 0.5 V cm$^{-1}$ is a potent migration cue for BTICs to move toward the anode with enhanced motility, and although cell motility did not further increase with increasing electric fields, directional bias progressively increased at a higher electric field.
**Figure 3-2 Characterization of the galvanotaxis of BTICs in 2D.**

(A). GBM612 cells clustered into neurospheres when cultured in suspension and stained positively for stem cell markers Sox 2 and nestin. (B and C) BTICs seeded on a glass surface coated with poly-L-ornithine and laminin in the absence (B) or presence (C) of a dcEF. In the absence of a dcEF, cells displayed random orientations with no directional bias. (C) In the presence of a 1 V cm⁻¹ dcEF, cells migrated preferentially toward the anode. Each colored trajectory represents the actual path traveled by a cell in three hours. (D) Immunostaining of cells immediately after exposing cells to a dcEF for 3 hours. The electric field induced cell protrusions in the direction of anode. The protrusions were abundant with microtubules in the center and actin filaments at the peripheral. (E and F) Overlay of the trajectories in the absence (E) and presence (F) of a dcEF showed the anodic response of BTICs on a PLO/LN substrate.
(G) Cell motility increased in the presence of a dcEF. Further increasing the EF from 0.5 V cm\(^{-1}\) to 1 V cm\(^{-1}\) did not significantly increase the motility. (H) An electric field as small as 0.5 V cm\(^{-1}\) was capable of biasing cell trajectories toward the anode. Cell directedness further increased with increasing electric field. * P < 0.05; ** P < 0.01; Student’s t-test. Statistics were obtained from at least three independent experiments with at least 60 cells in each experiment. Error bars indicate standard deviation.
3.2.2 Substrate charge dictates the direction of galvanotaxis

Galvanotaxis is a complex process involving interactions between physical and biochemical signaling. From the physical point of view, cells under a dcEF are mainly subjected to two types of forces: electrostatic and electro-osmotic forces. Charged particles, such as proteins and ions, are subjected to electrostatic forces in the presence of an electric field. The electrostatic interactions would then electrophoretically localized charged membrane components, such as EGFR, to one end of the cells and activate asymmetry signaling[49]. On the other hand, both charged and non-charged components are subjected to electro-osmotic forces generated by the charge associated with the cellular membrane or the underlying substrate. Therefore, to understand how various physical forces contributed to the galvanotaxis of BTICs, we first investigated the effects of electro-osmotic force on cell migration by modulating the charge of the underlying substrate.

BTICs are commonly cultured on a poly-L-ornithine (PLO) and laminin coated surface for better adhesion in serum free condition. PLO rendered surface to positively charged and thus created an electro-osmotic flow toward the anode in the presence of a dcEF (Supplemental Video 2*). It was hypothesized that the drag force generated by the electroosmotic flow would exert mechanical forces on adhesion complexes and integrins to displace cells laterally. We thus compared the galvanotaxis of BTICs on a PLO/laminin coated surface to cells on a surface coated with laminin only. Strikingly, although surface charge had no effects on the galvanotaxis of keratocytes [24], it had profound effects on the galvanotaxis of BTICs. Not only cells migrated in different phenotypes but the directions of galvanotaxis were opposite to one another on surface of different charges. On a PLO/LN coated surface, cells appeared more adhered and spread out and often migrated toward the anode with a fine single protrusion at the leading end.
(Figure 3-3A and Supplemental Video 1*), whereas on a laminin only surface, cells were more rounded and underwent frequent protrusion-retraction events as they moved toward the cathode (Supplemental Video 3*). In addition, cells migrated slower but more directional on PLO/LN surface with a mean directedness of -0.47 but faster and less directional on LN only surface (mean directedness = 0.32) (Figure 3-3). Immunostaining of the surface against laminin showed comparable fluorescent intensities between PLO/laminin and laminin only surfaces indicating the observed differences are not due to the variation in laminin density but surface charge (Figure S1). Similar results were obtained when laminin was replaced with type I collagen, where cells migrated toward the anode on a PLO/collagen surface but toward the cathode on a collagen only surface (Figure 3-3). This further suggested that surface charge plays an important role in determining the galvanotaxis of BTICs and the effect is independent of ECM type.

There are a couple ways surface charge may influence galvanotaxis: 1. Mechanical force associated with the electro-osmotic flow may “push” cells in the direction of migration. However, given the force calculated was on the order of pN and reversing the direction of flow with gravitational flow did not change the galvanotaxis response, Allen et al. concluded that electro-osmotic force is unlikely the driving force for galvanotaxis[24]. To rule out the possibility of electro-osmotic force being the dominating factor in the galvanotaxis of BTICs, we also carried out similar experiments and found that reversing the direction of drag force had no effects on the galvanotaxis of BTICs. As a result, electroosmotic force is unlikely the driving physical mechanism that governs galvanotaxis. 2. Electrostatic interactions between the substrate and the plasma membrane can mediate the balance between competing mechanisms that result in a cathodic or anodic response. Since most cell surfaces are negatively charged at physiological pH, cells seeded on positively charged substrates, such as poly ornithine, experience a strong
electrostatic attraction, which may impede the electrophoresis of cellular membrane components. In addition, interaction between the negatively charged glycocalyx surrounding the cells and the substrate may also influence integrin clustering and affects cell migration [50]. In fact, β4 integrin was known to be indispensable for the galvanotaxis of human keratinocytes [50]. Although surface charge-dependent galvanotaxis has never been reported previously, Xenopus spinal neurites were shown to undergo galvanotropism based on the sign of the surface charge: anodic growth on positively charge surfaces and cathodic on negatively charged surfaces [51]. The interplay between adhesion, surface charge, and cell motility thus become an interesting topic in understanding galvanotaxis.
Figure 3-3 Galvanotaxis of BTICs is highly regulated by substrate charge.

Cells migrated in different phenotypes and opposite directions on PLO/LN surface in comparison with LN only surface. Poly-L-ornithine rendered surface to positively charged and resulted in anodic galvanotaxis regardless of ECM types. Removal of PLO reversed directional response toward the cathode.
3.3 Involvements of heparan sulfates

Heparan sulfate (HS) is a linear polysaccharide ubiquitously expressed on all cell types. It belongs to the family of glycosaminoglycan (GAG) and often exists as a heparan sulfate proteoglycan (HSPG), where a core protein is covalently connected with one to five HS or other GAG chains. Due to its sulfation modification, HS is amongst the most highly negatively charged biopolymers in nature [52]. Membrane bound HSPGs are known to regulate many physiological events including cooperating with other integrins and cell adhesion receptors to facilitate cell motility and adhesion [53], serving as a coreceptors for various tyrosine kinase-type growth factor receptors [54], and acting as a endocytosis receptors for clearance of bound ligands and exosomes [55]. In glioma cells specifically, HSPGs were shown to stimulate tumor survival, growth, and invasion [56] [57]. The highly charged HS chains greatly increase the hydrodynamic volume of the proteins and extend the effective range of cellular interaction with ligands by a factor of three comparing to in the absence of HSPGs [58]. Since the electrical signal must first propagates from the cellular membrane and the cellular surface is abundant with highly charged HS chains, we began to wonder the involvements of HS on galvanotaxis.

3.3.1 Heparan sulfates are localized near the anode in the presence of an electric field

As expected, BTICs stained positively for heparan sulfate as shown by the punctate aggregates of HS observed at the lamellipodium (Figure 3-4A and Figure 3-5A). In the meantime, cells seeded on a neutral surface coated with laminin were also stained for HS after stimulated with a 1 V cm\(^{-1}\) electric field for six hours. In the presence of an EF, cells exhibited drastically different HS staining pattern as compared with the no electric field control; HSs localized at the anodal face opposite to the direction of cell migration (Figure 3-4B). To quantify the localization, a Wilcoxon signed-rank test was performed among the top 10% pixel intensities between the
cathode facing side (C) and the anode facing side (A). We found that electrical stimulation significantly increased the percentage of cells with a localization of HS at the anodal face: 73% of the cells (60 out of 82 cells) showed localization of HS toward the anode, whereas only 40% of the cells (30 out of 77 cells) in the control group showed anodic localization (Figure 3-4D).
Figure 3-4 Heparan sulfate are localized at the anodal face in the presence of an electric field.

(A) GBM612 cells stained uniformly for heparan sulfate (red) in the absence of an electric field. (B) In the presence of a 1 V cm\(^{-1}\) electric field, heparan sulfate got localized at the anodal face (trailing edge), while cells migrated toward the direction of cathode. (C) A representative gray scale image of the distribution of HS in the presence of an electric field. Localization of HS is analyzed by first dividing the cell down the geometric centroid into the cathode-facing side (\(C\)) and the anode-facing side (\(A\)), and then performing a Wilcoxon signed-rank test between the pixel intensities within \(C\) and \(A\). (D) A significantly higher percentage of cells showed anodic localization of HS (73%) in the presence of an electric field than cells under no electric field (40%).
3.3.2 Enzymatic removal of HS reversed the directional response of BTICs

To investigate the roles of HS on the directional response of BTICs, GBM612 cells were treated with 2 sigma-unit (S.U.) per milliliter of Heparinase (HPase) I and III and 500 mU chondroitinase ABC (chABC) to cleave off the HS chains and chondroitin sulfate chains from the membrane. HPase treatments cleaved off the HS chains found on the cellular membrane leaving significantly less or no punctate HS aggregates on cell processes (Figure 3-5B) as compared to cells without the treatment (Figure 3-5A). Moreover, when exposed the HPase treated cells to a 1 V cm\(^{-1}\) dcEF, cells migrated at a similar motility to wild type but in a completely opposite direction (Figure 3-5C,D, and F); HPase treated cells seeded on a neutral and laminin-coated surface migrated toward the anode (Figure 3-5D), whereas wild type GBM612 cells migrated toward the cathode on the same surface (Figure 3-5C).

3.3.3 PLO/LN surface prevents redistribution of HS in the presence of an electric field

BTICs cultured on a PLO/LN surface in the presence of a dcEF were also stained for HS to see whether the electrostatic interaction between cells and the substrate would interfere with the electrophoresis of HS. In the presence of an electric field, not only did cells migrate toward the anode at a much lower motility (Figure 3-5F), but also no localization of HS was found. On a PLO/LN surface in the presence of an electric field, punctate HS aggregates were observed on the cellular processes with uniform distribution (Figure 3-5E), and patches of HS can be found deposited on the substrate at the trailing edge of the cell as the cell migrated toward the anode (Figure 3-5E, arrow). The presence of a positively charged PLO not only enhanced cellular adhesion and restrained cell motility but also prevented HS from redistributing under the influence of the electrophoretic force.
Figure 3-5 Localization of HS to the anode is required for the cathodic directional response.

(A) GBM612 cells stained positively for heparan sulfate as shown by the punctate aggregates located at the lamellipodium and migrated toward the cathode when seeded on a laminin-coated neutral surface (C). (B) Treatments with a blend heparinase I and III largely removed the amount of HS on the cellular membrane as shown by the very limited amount of punctate aggregates at the lamellipodium. More importantly, removal of HS led to a reversal in directional response; cells migrated toward the anode when treated with heparinase (D). (E) Addition of a positively charged polymer layer on the substrate provided additional electrostatic force and abolished
redistribution of HS. The positively charged PLO layer substantially attracted and anchored HS chains onto the substrate and thus, trails of HS could be found left along the migration path (arrow). (F) Treatments with heparinase or rendering the substrate to be positively charged led to anodic migration. However, heparinase treatments did not affect cell motility while the addition of PLO significantly decreased cell motility possibly due to the enhanced cellular adhesion resulted from electrostatic attraction.
3.4 HSPG as an electric field sensor responsible for cathodic migration

The significantly higher percentage of cells with HS localized near the anode is likely due to electrophoresis in response to the applied electric field, where highly negatively charged HSs got attracted toward the positive end. In human keratinocytes, epidermal growth factor receptors (EGFRs) were shown to concentrate on the cathodal face of the cell as early as five minute after exposure to an electric field and inhibiting EGFR kinase activity abolished directional response and localization [59]. However, given that EGFR has an isoelectric point of 6.26, it is baffling how EGFR got localized at the cathodal face. Whether the localization of EGFR is a direct consequence of an electric field or a secondary effect has yet been understood. Considering the charge and direction of polarization, we believe the localization of HS is much more likely a direct consequence of the applied electric field.

Using human keratinocytes, Allen et al. previously showed that galvanotaxis of keratinocytes is insensitive to membrane potential change and perturbations of many types of ion transports across the membrane; however, lowering the pH or increasing liquid viscosity significantly attenuated the cathodic response of cells, indicating that the electrophoresis of charged membrane components is the dominating mechanism for galvanotaxis[24]. Through a combination of immunofluorescence visualization and enzymatic treatments, we showed here that HSPG is an electric field sensor responsible for the cathodic response of BTICs, and it may very likely be the elusive component responsible for the directional response of keratinocytes and other cell types.

The fact that both enzymatically cleaving HS off the cellular membrane and electrostatically changing the charge of the underlying substrate both yield anodic response imply the following mechanisms of action as illustrated in Figure 3-6: 1. Competing mechanisms responsible for
anodic response exist, and the final direction of migration is a tug-of-war between HSPG-facilitated cathodic response and the counteracting anodic response, which has yet to be identified. 2. Asymmetric distribution of HSPG to the trailing edge (anode) is facilitated by electrophoresis of HS chains and required for the cathodic directional response of BTICs; failure of relocating HS to the anode due to additional electrostatic interaction tilt the balance in favor of anodic directional response.

3.4.1 Syndecan as a possible HSPG candidates to regulate cathodic response

Visualizing the distribution of antibody-labeled HS, we showed that electrophoresis of HS to the anode is critical for the cathodic response of BTICs. However, how the relocation of HS to the trailing edge leaded to the directional migration in the opposite direction remained an intriguing question. Here, we propose a possible mechanism in which the relocation of HSPG to the anodal face suppresses Rac1 activity and thus indirectly promotes cell migration toward the cathode (Figure 3-6A). Since HS chains are covalently linked to its core protein in forming HSPG, it is very likely that electrophoretic localization of HS also redistributes HSPG to the trailing edge and establishes cell polarity. Two specific HSPG candidates capable of being such modulator are Syndecan-4 (Syn4) and Syndecan-1 (Syn1).

3.4.2 Possible roles of Syndecan-4 (Syn4) in regulating the cathodic response

Syndecan-4, isoelectric point of 4.39, is known to regulate directional cell migration both in vitro and in vivo [53] [60]. In vitro, Syn4 was shown to mediate persistent migration of wild type fibroblasts by localizing Rac1 activity and membrane protrusion to the leading edge of the cell in response to extracellular matrix, whereas Syn4-null fibroblasts migrate randomly due to highly delocalized Rac1 activity [53]. In vivo, Syn4 is critical for proper directional migration of neural crest cells. During embryonic development, Syn4 together with Wnt/PCP signaling
collectively inhibit Rac1 activity at the trailing edge to regulate the direction in which
protrusions are generated [60]. However, despite Syn4 was shown to inhibit local Rac1 activity
to establish polarity, it remains baffling how Syn4 got localized at the trailing edge to determine
the direction of migration during embryogenesis. Our observation where HS are localized at the
trailing edge of a cell in the presence of an EF might precisely explain such question.

We hypothesized that Syn4 in the presence of an electric field got localized toward the anode due
to the electrophoresis of highly negatively charged HS chains. As a result of the localization,
Syn4 asymmetrically inhibits Rac1 activity at the anodal face, which in turns promotes cell
migration toward the cathode (Figure 3-6A). Perhaps similar scenario cab be applied to the
directional migration of neural crest cells during embryo development. In fact, multiple studies
have shown that endogenous electric fields are important during embryogenesis and disrupting
the local electric field often leads to developmental abnormality [61][62]. Interestingly, Syn4
deficient mice also exhibited defects in wound healing [63]. Considering the following
conditions: 1. Galvanotaxis is heavily implicated during wound healing [64] and 2. Lack of Syn4
resulted in defects in wound healing [63], we believe Syn4 is a compelling candidate responsible
for the regulation of galvanotaxis.

3.4.3 Syndecan-1 (Syn1) as a candidate for regulating cathodic response

In contrary to Syndeca-4, which has a ubiquitous expression, Syndecan-1, isoelectric point of
4.26, has a restricted tissue distribution and has gained a lot of attention recently due to its close
link to various progressions of disease including inflammatory disease, infection, and cancer
[65]. For example, Syn1 was known to activate integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ to mediating cell
adhesion and angiogenesis, and blockage of Syn1 significantly affected angiogenesis and
tumorgenesis in mouse models [66]. Expression of Syn1 in human glioma was also known to
correlates with the advance of tumor progression [67]. More importantly, Syn1 was also known to negatively modulate cell motility by either interacting with integrins to affect the formation of focal adhesions or by directly influencing actin cytoskeletons [68, 69]. Knocking down the expressions of Syndecan-1 via siRNA resulted in an increase in Rac1 activity and cell motility. Together, these studies make Syn1 another promising candidate in regulating the cathodic response of GBM612 cells.
Figure 3-6 Schematic illustrations of possible mechanisms regulating a cathodic response on a neutral surface and an anodic response on a positively charged PLO-coated surface

(A) Heparan sulfate localized toward the anodal face while the cell migrated in the opposite direction toward the cathode on a neutral surface coated with laminin. The columbic force exerted on the HS chains also led to the electrophoresis of HSPGs to the anodal face which triggered an asymmetric signaling of actin polymerization. Syndecan, as one of the only two types of membrane-bound HSPG, has been shown to regulate Rac1 activity for persistent migration both \textit{in vivo} and \textit{in vitro}, and therefore, is a prime HSPG candidate that regulates
cathodic response. (B-C) Disrupting the localization of HS leads to anodic response. (B)

Enzymatically removing HS chains on the cellular membrane greatly reduced the coulombic force
exerted on the HSPG, and thus, inhibited the localization of HSPG toward anode. (C) The
addition of a positively-charged polymer layer electrostatically immobilized HS from relocating.
The fact that the galvanotaxis of BTICs can be either cathodic or anodic indicates that at least one
competing mechanism that drives cells to migrate toward the anode exists, and disrupting HS
localization tilted the balance in favor of an anodic response.
3.4.4 Co-localization of HS with Syn1 but not Syn4 in the presence of an EF strongly suggested that Syn1 is a key regulator for cathodic response

So far we have shown that HS localized at the anodal face, yet the identity of the HSPG corresponded to the localization is still illusive. To uncover the identity of the HSPG, cells stimulated with an dcEF were simultaneously stained for HS, Syn4, and Syn1 (Figure 3-7). From the immunofluorescence staining, we showed that HS did not co-localize with Syn4 (Figure 3-7B), but instead, co-localized with Syn1 at the anodal side (Figure 3-7D). This finding strongly supported our hypothesis where the Columbic force exerted on the negatively charged HS resulted in the electrophoresis of the HSPG, in this case, Syndecan 1 to the anodal face. Since Syn1 was shown to upregulate Rap1 and promoted cell contractility through RhoA [69], we believe the localization of Syn1 would inhibit the activity of Rac1 at the anodal face, which in turns promoted cells to migrate toward the cathode.
Figure 3-7 Co-localization of HS and Syn1 at the trailing anodal face strongly suggested the important role of Syn1 in regulating cathodic response

(A) Heparan sulfate got localized at the anodal face while the cell migrated toward the cathode in the presence of an electric field. (B) Syndecan-4 did not co-localize with HS in the presence of an EF and predominately expressed close to the cellular nucleus. (C) Syndecan-1 localized at the trailing edge of the cell in the presence of an EF and showed a nearly identical staining pattern as HS. (D) Overlay of HS and Syedcan-1 staining patterns (yellow) showed an exact co-localization between HS and Syn1 at the trailing edge while Syndecan 1 is slightly more expressed at the leading edge as shown by the predominately green pattern at the front.
3.5 Mechanisms responsible for anodic response

We have showed that competing mechanisms responsible for cathodic and anodic responses exist, and the electrophoresis of Syndecan-1 toward the anodal face is very likely the dominating factor for cathodic response. Several downstream signaling mechanisms responsible for cathodic migration have been identified. For example, the PI3K-dependent pathway was shown to be associated with cathodic response in keratinocytes, where the inhibition of PI3K resulted in the reversal of directional response from cathodic to anodic [24]. In addition, genetic manipulation of guanylyl cyclases (GCases) and the cyclic guanosine monophosphate (cGMP)-binding protein C (GbpC) in combination with the inhibition of phosphatidylinositol-3-OH kinases (PI3Ks) in *Dictyostelium* cells also resulted in the reversal of directional response [44]. However, despite intensive research, the signaling mechanisms underlying anodic response is still largely elusive. Thus, we also attempted to explore the anodic signaling mechanisms by performing a series of drug inhibition assay on GBM612 cells culturing on a PLO/LN-coated surface, where the default direction of galvanotaxis is toward the anode.

3.5.1 Anodic response of BTICs is not solely dependent on any small-GTPases

Rho family of GTPases, such as Cdc42, Rac, and Rho, are known to relay external signals and regulate actin cytoskeletons and microtubule dynamics that are important for effective long range cell migration [70]. To have a better understanding of the galvanotaxis of BTICs, we used a series of pharmacological perturbations against actin filaments, microtubules, and Rho family of GTPases in the presence of a dcEF. Although motility of glioblastoma cells was reported to occur in the absence of actin polymer previously [71], inhibiting actin polymerization with 250 ng ml⁻¹ of latrunculin A significantly down regulated the motility of BTICs (*Figure 3-8*). On the other hand, disruption of microtubules with 200 ng mL⁻¹ of nocodazole also significantly
attenuated cell motility (*Figure 3-8*). This was not surprising given the protrusions of BTICs in the presence of a dcEF were abundant with microtubules (*Figure 3-8*). However, in both cases, cell migration still remained biased toward the anode, which indicated that BTICs require both actin filaments and microtubules to migrate effectively and both components contributed to the directional response in 2D.

BTICs were further treated with Rho GTPases inhibitors to understand their involvements in galvanotaxis. Selective inhibition of Cdc42 with 50μM of ZCL278 had no effect on either cell motility or directedness (*Figure 3-8*). This was quite surprising, since 50μM of ZCL278 was shown to suppress the formation of filopodia and significantly suppressed cell migration into the wound area in human prostate cancer cell line (PC-3)[72]. Disruption of Rac1 with 50μM of NSC23766 or Rho-associated protein kinase (ROCK) with 50μM of Y27632 significantly down regulated cell motility to 0.31 (p=0.046) and 0.26 μm min⁻¹ (p=0.03) respectively. Although perturbations of Rac had no effect on directedness, cells treated with ROCK inhibitor showed an increase in directedness unexpectedly. These results are in contrast with data previously reported in the literature, where inhibiting Rac1 completely abolished galvanotaxis in human keratinocytes [73] and inhibiting ROCK significantly increased cell motility but reduced directedness by 70-80% in human induced pluripotent stem cells [74]. Overall, we showed that pharmacological perturbations of Cdc42 and Rac had no discernible effect on the directedness of the cells in the presence of a dcEF, whereas ROCK negatively regulated cell directedness in 2D. Disrupting Rac1 and ROCK down regulated cell motility, whereas interfering Cdc42 had no effect on motility.
Cell migration is considered as a process highly coordinated between each Rho-family of GTPases, where Cdc42 is responsible for the formation of filopodia and maintenance of cell polarity, Rac1 is important for the accumulation of actin and formation of lamellipodia, and Rho regulates the assembly of contractile actin-myosin filaments [70]. While it makes sense that perturbations of Rac and Rho impaired motility as cell migration depends on effective adhesion and detachment, it remained perplexing how disruption of Cdc42 failed to affect galvanotaxis as Cdc42 is critical for polarization and directional migration during chemotaxis[75]. One possible explanation may be that directional sensing of galvanotaxis is more of a physical process, where electrophoretic or electroosmotic force provided a physical guidance similar to the case in shear stress response. For example, shear stress-induced polarization of endothelial cell was shown to be mediated by Rac and Rho but not Cdc42 or PI 3-kinases [76]. Furthermore, the signaling pathways for the reception and transduction of signals were shown to be independent of each other between galvanotaxis and chemotaxis in Dictyostelium discoideum[77]; it is possible that the downstream signaling strategies are also different, which would explain the different role of Cdc42 in galvanotaxis versus chemotaxis.
Drug screening assay reveals galvanotaxis of BTICs is negatively regulated by ROCK and MAPK but dependent on the activation of PI3K.

Inhibition of actin filaments or microtubules significantly down regulated cell motility without abolishing directional response. Blocking either Rac or ROCK down regulated cell motility (p=0.016 and 0.03 respectively), whereas inhibiting Cdc42 had no significant effect on galvanotaxis. Interestingly, inhibition of either ROCK or MAPK, significantly increased directedness of cells (p=0.017). Disruption of PI3K significantly down regulated directedness (p=0.009) but had no effect on cell motility. Pharmacological perturbations of known receptors involved in the chemotaxis of glioblastoma including EGFR, PDGFR, VEGFR2, CXCR4 and
CXCR2 had no effects on either cell motility or directedness. * P < 0.05; ** P < 0.01; Student’s t-test. Statistics were obtained from at least two independent experiments with at least 50 cells in each experiment. Error bars indicate standard deviation.
3.6 Galvanotaxis in 3D

To further study the galvanotaxis of BTICs in a more physiological environment, cells were next embedded in a 3D ECM matrix made of hyaluronic acid and collagen then stimulated with a physiological relevant electric field. Hyaluronic acid was selected as it is the most abundant ECM in the brain and was shown to be critical in maintaining the homeostasis inside the brain [78], whereas although the brain is largely devoid of fibrillar proteins, collagen provides the necessary cellular support and adhesion sites without altering the stiffness of the gel at low concentration (1 mg mL⁻¹) [23]. Various groups have highlighted the striking differences between 2D and 3D migration in the past[79]. For example, to effectively migrate in a 3D matrix, glioma cells are required to degrade the ECM via matrix metalloproteinase and squeeze through tortuous confinements with constant deformations and strong retraction forces, but neither of these is required in 2D.

Here we have also observed great differences between 2D and 3D migration of GB612 cells. In 2D, migration of 612 cells is characterized by the stable formation of lamellipodium at the leading end and the retraction of cellular processes at the trailing end (Supplemental video 1*). However, in 3D cells adopted completely different phenotypes. Not only lamellipodium were not observed in 3D, cells were also devoid of trailing processes. Instead, cells migrated with a single long pseudopodia at the leading front and underwent very dynamic protrusion cycles (Supplemental video 5 and 6*). In the presence of a dcEF, directedness was strongly biased by the electric field; cells extended characteristic protrusions toward the cathode followed by contraction of the cell bodies to propel themselves (Figure 3-9A). Cell bodies also frequently deformed into “hour glass” shapes as cells squeezed through physical confinements. Analysis of cell trajectories indicated that both cell motility and directedness significantly increased in the
presence of a 1 V cm\(^{-1}\) field \textcolor{blue}{(Figure 3-9B and C)}. Comparing to 2D migration on a collagen and hyaluronic acid coated surface, cell exhibited lower motility in 3D primarily due to the requirements to degrade surrounding ECM, but the directedness was largely unaffected in 3D \textcolor{blue}{(Figure 3-9D)} indicated that galvanotaxis may also be a potent migration cue even in a physiological relevant three-dimensional ECM..

Lastly, we looked at the effect of inhibiting PI3K on 3D galvanotaxis. To ensure the compound was uniformly distributed within the ECM, 50\(\mu\)M of LY294002 was mixed with the cells and ECM prior to introducing into the device. Interestingly, in contrary to the results observed in 2D, cell motility was lower in the presence of PI3K inhibitor in 3D, whereas the directedness significantly increased. This further indicated the biological and physical differences in 3D vs 2D migration. Interplay between cells and the surrounding environment can affect the involvement of different signaling pathways.
**Figure 3-9 Galvanotaxis is a potent migration cue for BTICs in 3D.**

(A) Cells embedded in ECM made of a mixture of 1 mg mL⁻¹ of collagen and hyaluronic acid exhibited unique phenotypes and migrated toward the cathode in the presence of an dcEF. (B) Overlay of cell trajectories in the absence and presence of a dcEF indicated a strong cathodic response in the presence of an electric field. (C) Cell motility only significantly increased at 1 V cm⁻¹, whereas directedness progressively increased with increasing field strength. (D) Cell motility is slightly hindered in 3D but the directedness remained similar to 2D. Inhibiting PI3K further down regulated cell motility in 3D; however, comparable directedness were found between 2D, 3D, and even after inhibiting PI3K in 3D. * P < 0.05; ** P < 0.01; Student’s t-test. Statistics were obtained from at least three independent experiments with at least 50 cells in each experiment. Error bars indicate standard deviation.
3.7 Conclusion

We have shown here that electric fields as small as 0.5 V cm\(^{-1}\) is a prominent directional migration cue for glioblastoma cells. The direction of galvanotaxis is indeed a tug-of-war between competing mechanisms where the balance is tightly hinged on the electrophoresis of HSPG. We have not only identified HS chains as a novel extracellular electric field sensor but also pin pointed Syndecan-1 as the key regulator for cathodic response in both 2D and 3D migration. Despite the mechanisms for anodic response is still largely unknown, through a panel of chemical inhibitions, we showed that PI3K is partially involved in the response. Collectively, this work discovered the first directional sensing mechanism utilized by cells and provided valuable insights in understanding the complexity of galvanotaxis.
3.8 Supporting informations

Figure S1. Concentrations of laminin at different coating conditions

Glass coverslips coated with laminin only, poly-L-ornithine (PLO)/laminin, and PLO/laminin (2X, doubled concentration) showed similar fluorescent intensity when stained with laminin antibodies. The similarity in fluorescent intensity indicates that addition of PLO does not affect the amount of laminin adhered on the surface, and that the surfaces have already been saturated with laminin.
Chapter 4 Future experiments and outlook

Electrical migration cue provides several unique advantages that are unparalleled by other migration cue. For example, the easiness and steadiness of establishing an electrical gradient allow precise temporal control of cell migration. Yet, in the meantime, the lack of specificity and difficulty for in vivo integration also pose a steep challenge. Our future direction of research can be classified into two categories: one from the mechanistic perspective and the other from the perspective of translational science.

4.1 Mechanistic perspective

We showed both HS and Syn1 got localized toward the anodal face and that HS is indispensable for the cathodic response of GBM612 cells. Therefore, we would like to further confirm the necessity of Syn1 by suppressing its expression using siRNA. We expect cells to be in favor of anodic migration in the absence of Syn1; however, Syn1 may also be crucial for the migration machinery and therefore significantly suppress cell motility when its expression is down-regulated. In addition, we are also interested in over-expressing Syn1 and see whether the expression of Syn1 correlates with the directedness of the cells in the presence of an electric field. It has been shown that nerve injury induces the expression of Syn1 in peripheral motor neuron, and it will be interesting to see whether this evolutionary consequence is related to galvanotaxis to aid the repair of neurons. We are also planning on testing whether our findings can be generalized toward other glioblastoma or other cell lines.

4.2 Translational applicability

We previously showed that cellular environment plays an important role in regulating galvanotaxis. For example, 1D confinement significantly increased cellular motility of fibroblast,
whereas substrate charge in 2D dictates the direction of galvanotaxis in glioblastoma cells. As a result, we believe both motility and direction of galvanotaxis can be fine-tuned depending on the applications. The possibility of combining topographical cues such as using conductive nanofibers and electrical and other migration cues is rarely explored and may be translated into many biomedical applications in the future.
Chapter 5 References


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