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Directed cell migration in multi-cue environments

Abstract

Cell migration plays a critical role in development, angiogenesis, immune response, wound healing and cancer metastasis. During these processes, cells are often directed to migrate towards targets by sensing aligned fibers or gradients in concentration, mechanical properties or electric field. Often times, cells must integrate migrational information from several of these different cues. While the cell migration behavior, signal transduction and cytoskeleton dynamics elicited by individual directional cues has been largely determined, responses to multiple directional cues are much less understood. However, initial work has pointed to several interesting behaviors in multi-cue environments, including competition and cooperation between cues to determine the migrational responses of cells. Much of the work on multi-cue sensing has been driven by the recent development of approaches to systematically and simultaneously control directional cues *in vitro* coupled with analysis and modeling that quantitatively describe those responses. In this review we present an overview of multi-cue directed migration with an emphasis on how cues compete or cooperate. We outline how multi-cue responses such as cue dominance might change depending on other environmental inputs. Finally, the challenges associated with the design of the environments to control multiple cues and the analysis and modeling of cell migration in multi-cue environments as well as some interesting biological questions associated with migration in complex environments are discussed. Understanding multi-cue migrational responses is critical to the mechanistic description of physiology and pathology, but also to the design of engineered tissues, where cell migration must be orchestrated to form specific tissue structures.

Keywords

chemotaxis, haptotaxis, durotaxis, mechanotaxis, contact guidance, electrotaxis, galvanotaxis

Disciplines

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Directed Cell Migration in Multi-cue Environments

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galvanotaxis

sin II activity and is transmitted through adhesions⁸. Through careful coordination between protrusion at the front of the cell and attachment at the rear, retraction follows and the trailing edge slides forward⁹. An additional step of proteolytic cleavage of the dense ECM is required in most 3D matrices.

Cells move either individually or collectively¹⁰ and these modes of migration function in different biological contexts. For instance, individual cell migration drives cell trafficking and immune function¹¹, collective cell migration governs development and organogenesis¹², and either individual or collective cell migration promotes cancer invasion and metastasis¹³. Individual cell migration can be described as amoeboid (proteinase independent and contractility dependent) or mesenchymal (proteinase dependent and contractility independent). In contrast to individual cell migration, collective cell migration occurs when cells adhere strongly to each other and move as a group¹⁴. When cells transiently interact through cell-cell contacts, cell streaming occurs and represents a mixed phenotype consisting of characteristics of both individual and collective cell migration. Although the underlying processes mentioned in the previous paragraph are used during both individual and collective cell migration, noticeable differences exist. First, during collective cell migration the maintenance of cell-cell adhesion can hinder the migratory activity of the cells inside the group, but does not seem to affect a certain population of cells involved in translocating the group. Second, during collective migration certain cells are selected for specialized functions resulting in or caused by differences in gene expression¹⁵, signal transduction¹⁶ and actomyosin dynamics¹⁷. These different cells are often referred to leader or tip and follower or stalk cells and are important during processes such as angiogenesis¹⁸. The tissue environment and cell state define whether cells migrate individually or collectively. While collective migration certainly is important and is no doubt driven by the

environmental factors discussed in this review, we will primarily focus on individual cell migration for the remainder of the article.

In the absence of a directional cue and over timescales longer than the timescale of polarization the processes of protrusion, adhesion, contraction, traction generation, retraction and proteolysis occur randomly throughout the cell. Additionally, if a migrational cue is homogeneously distributed, the rates of these processes can increase. However, if there is a directional cue, these processes can be biased in particular regions of the cell leading to directed migration. We will first give a brief overview of quantitative measurements of random cell migration as well as some governing principles that regulate random cell behavior. We will then devote more time to the quantitative measurements of directed cell migration, engineered environments with the capacity to spatially present migrational cues and some governing principles that regulate directed cell migration.

Random Cell Migration:

Different mathematical models have been proposed to describe random cell migration. For instance, the movement of single cells has been analyzed using a persistent random walk model which assumes that the mean squared displacement depends only on speed (S) and persistence time (P). Speed is the displacement over a short time interval and persistence time characterizes the average time over which changes in direction and speed are insignificant¹⁹. The speed and persistence time can be combined to form a random motility coefficient (μ), which is analogous to a diffusion coefficient. Alternatively, cell migration has been characterized by run and tumble models, where cell trajectories are modeled as an alternating random sequence of movements in which changes in directions can be gradual or abrupt. Run and tumble movement is characterized

by tumbling frequency (f), run duration (t_{run}) and turn angle (θ)²⁰. A list of common approaches to quantifying random migration is presented in Table 1. All of these parameters are seen as constants under a given set of conditions. However, when extracellular cues are presented uniformly and at different concentrations, they impact cell migration by changing the above parameters.

The effects of uniform doses of migrational cues on random cell motility have been widely studied. For instance, a biphasic dependence of cell migration speed on the density of ECM, such as fibronectin or collagen has been observed²¹. At low ECM density, cells adhere weakly and cannot gain traction, whereas at high ECM density, cells adhere strongly and cannot overcome the adhesion through intracellular contraction. This optimal ECM density can be altered by tuning integrin affinity or number and drives optimal migration speed through a specific structural and dynamic organization of adhesions and the actin cytoskeleton²². Consequently, tuning the structure and dynamic organization of the adhesions and actin cytoskeleton either by pharmacological inhibitors of contractility or through the ECM with different mechanical properties can change the ECM density that gives optimal migration speed. If contractility is decreased the optimal ECM coverage occurs at lower densities and if contractility is increased the optimal ECM coverage occurs at higher densities²². Conversely, at one ECM density there exists an optimal substrate stiffness, which leads to an optimal level of contractility, resulting in a maximal migration speed²³. Similar biphasic observations have been noted when studying cell responses to a uniform concentration of soluble factors, such as growth factors²⁴. Growth factors not only control adhesion and contractility and thus can influence migration in a similar way to ECM density or mechanics, but also increase protrusion rates as well²⁵.

Understanding how cell migratory parameters such as cell speed depend on the ECM properties or concentration of soluble factors provides some general principles of cue-mediated random cell migration that can be applied to directed cell migration. For instance, ranges of ECM density, stiffness or soluble factor concentration that constitute significant migrational speed when cues are presented homogeneously apply when presented inhomogeneously. While directed cell migration undoubtedly uses the cell motility cycle, signaling pathways and the cytoskeleton, additional parameters must be defined to describe the spatial distribution of cue and bias in migration (Table 1). Additionally, directed migration requires more sophisticated approaches that allow for the control over the presentation of pro-migratory cues.

Directed Cell Migration:

When a cell is under the influence of a spatially inhomogeneous cue, the basic motility machinery is activated in a biased fashion leading to directed migration²⁶. The nature of the environmental cue defines the type of directed cell migration (Figure 1A). Contact guidance usually describes migration along the long axis of ECM fibers. On the other hand, haptotaxis, durotaxis (mechanotaxis), chemotaxis and galvanotaxis (electrotaxis) is the directed migration in response to gradients in ECM density, ECM mechanical properties, soluble factor concentration and electric field, respectively. These diverse types of directed cell migration are utilized under various physiological and pathological processes.

For instance, contact guidance is relevant in a variety of situations *in vivo*. During wound healing fibroblasts migrate efficiently along collagen or fibronectin fibers in connective tissues²⁷ and recent evidence has shown that ECM remodeling leading to aligned fibers of collagen oriented radially from the tumor is a good prognostic indicator of tumor invasive potential²⁸.

The most well-established role for haptotaxis *in vivo* is in T lymphocyte recruitment on endothelial cells to sites of infection. This occurs through sensing gradients in endothelial surface adhesion proteins such as PECAM-1²⁹. Haptotaxis has also been tenuously linked to angiogenesis and cancer invasion through gradients in collagen and laminin³⁰. Durotaxis or mechanotaxis may be important in blood vessel development. In addition, there appears to be a clear relationship between ECM stiffness and cancer progression³¹. However, the dependence on gradients in mechanical properties per se has also not been well established. Chemotaxis on the other hand has been largely implicated in wound healing, immune response and cancer metastasis. Degranulating platelets are known to secrete platelet-derived growth factor (PDGF) that diffuses from the provisional clot into the surrounding tissue, recruiting dermal fibroblasts who chemotax in response to PDGF^{24b}. In addition, neutrophils migrate up gradients of fMLP, a peptide released by bacteria during immune response³². Finally, researchers have recently established a paracrine loop between certain cancer cells and immune cells whereby each secretes chemoattractants for each other. Consequently, cancer cells have been shown to migrate towards and into the tip of a pipette releasing epidermal growth factor, a known chemoattractant for breast cancer cells³³. Galvanotaxis (electrotaxis) has been implicated in wound repair as well. Evidence shows that the disruption of epithelial layers that surround organs or compose the epidermis generates a steady voltage across the wound site and consequently a lateral electric field³⁴, suggesting that cells electrotax during the initiation of tissue regeneration processes.

Contact Guidance:

Contact guidance has been shown to drive migration through topographical features of the ECM³⁵. Most commonly, contact guidance describes the directed migration biased by aligned fibers or

fiber-like geometries. First evidence of this phenomena was noted by Weiss³⁶ in connective-tissue cells cultured on fibers, grooves or strand architectures. Contact guidance is driven by the fiber density and the degree of alignment measured as a distribution of cell-bound fiber angles. If the distribution is large, cells will have a more difficult time interpreting the mean angle of orientation. At low degrees of alignment, the fibers are isotropically oriented and random migration should occur separate from fiber density. At some threshold of degree of orientation, cells should sense the directional cue and bias their migration towards that orientation. Individual or perfectly aligned fibers or fiber-like structures can very persistently orient cell migration^{27a} with migration speed highly dependent on the type of contact guiding cue. What is not known is if a threshold in degree of alignment is needed for biased migration and how the contact guidance index differs as a function of the degree of alignment, two important questions given fibers are not usually perfectly aligned *in vivo*. In addition, fiber orientation thresholds for contact guidance are most likely functions of fiber density. Indeed, microcontact printing suggests that directed migration might rely on the spacing of binding sites along the length of the fiber with respect to the fiber-to-fiber spacing³⁷. The mechanism of sensing fiber direction most likely occurs at the level of alignment of focal adhesions and the actin cytoskeleton.

Different approaches have been reported to present contact guidance signals, but most seek to generate fiber or fiber-like topology. Microcontact printing is a protein deposition technique that can be used to transfer 2D patterns onto a surface. Patterns can be made to print lines of ECM and this technique has been used extensively to characterize cell orientation and migration^{27a, 38}. However, lines of ECM do not completely recapitulate fiber structure and the degree of alignment of fibers cannot be easily tuned, so other methods have been employed including electrospinning³⁹ and other fiber forming techniques⁴⁰. These techniques allow for the

control over fiber orientation and can be used with ECM polymers like collagen. In addition to these techniques, collagen fibers can be deposited as a thin film or epitaxially grown on mica to generate oriented fiber fields⁴¹. Finally, techniques to orient fibers, particularly collagen fibers in 3D matrices have been developed and use magnetic fields or flow for fiber alignment⁴².

Haptotaxis:

Haptotaxis or biased migration in response to surface bound ligand gradients was first established in mouse fibroblasts⁴³. As mentioned previously, the mean ECM concentration has a tremendous influence on the level of integrin-ECM adhesive interaction, which controls cell migration speed^{21b,44}. Consequently, while the gradient steepness affects the degree to which migration is biased, the average ECM density regulates migration speed setting concentration ranges that produce the fastest migration speeds. A recent study showed that the drift velocity of cells on well-defined haptotactic gradients increased linearly with fibronectin gradient magnitude⁴⁵. Additionally, the threshold for gradient sensing was ~3-20% with saturation in directionality occurring at 30-60%.

Throughout the years, new methods to modify surfaces and generate gradients have been developed. Early studies use Boyden chambers that allowed the migration of cells across a porous membrane previously coated with ECM proteins on its lower side³⁰. However, Boyden chambers have lost popularity due to the inadequate control of surface concentration and the indirect measurement of cell migration. Alternatively, other reports explore the use of self-assembled monolayers (SAMs)⁴⁶ or polymer brushes to control ligand deposition⁴⁷. The development of microfluidics and micro fabrication technologies has fostered their implementation to generate surfaces with a gradient of ECM bound proteins⁴⁸. Very recently,

adhesive gradients were also fabricated in scaffolds of electrospun fibers by modulating the rate and concentration of polymer solution and adhesive peptide incorporated during fabrication ⁴⁹.

Durotaxis (Mechanotaxis):

Durotaxis (mechanotaxis) corresponds to directed migration towards regions of increased stiffness ⁵⁰. Both stiffness magnitude and gradient drive cell migration. As detailed previously, the link between the stability of focal adhesions and the ECM stiffness was showed by studies examining random migration. This most likely leads to optimal migration speeds at intermediate stiffness. However, the relation between a stiffness gradient and directed cell migration has not been explored until very recently, based on experiments in which on polyacrylamide substrates with an induced stiffness gradient migrated consistently in the direction of increased stiffness.

To generate the stiffness gradient, polyacrylamide gels with different amounts of crosslinking agent but equal chemical composition have been used ⁵⁰⁻⁵¹. In these experiments, stronger traction forces on stiff substrates led to retraction when the cell encountered soft substrates. Moreover, epithelial cells cultured on microfabricated substrates that exhibit anisotropic stiffness (one direction stiffer than the other) were oriented and migrated in the direction of greater stiffness ⁵². It was concluded that the anisotropic growth (and consequently migration) of cells was correlated with the mapping of the mechanical traction forces exerted by the cell and the actin cytoskeleton orientation. However, unlike haptotaxis, much less is known about the degree of migrational bias as a function of the steepness of the gradient in mechanical properties.

Chemotaxis:

Chemotaxis is the phenomenon by which the cell migrates up a gradient of soluble factor⁵³. In a similar way to haptotaxis, chemotaxis is most efficient at intermediate concentrations of chemoattractant. This intermediate concentration is related to the K_d of the receptor and is near the saturation point of the signal transduction cascade^{4e, 54}. In addition, some cells like neutrophils are exquisitely sensitive to shallow chemoattractant gradients around 1-2%^{54b}. Whereas other cells like fibroblasts are less sensitive^{54a}. This is most likely due to the different ligand-receptor pairs, which determines the response of the signaling network. This response might include adaptation and cooperativity or ultrasensitivity to ligand doses. In addition to the steepness and midpoint concentration of chemoattractant gradients, nonlinearity in gradient shape may also affect the efficiency of chemotaxis⁵⁵.

Boyden, Zigmond and Dunn chambers were initially conceived using a source/sink design in which chemotaxis is characterized by either observing cell motion or measuring the net movement of cells towards the source^{54b, 56}. Other methods generated gradients using micropipettes⁵⁷, under-agarose assays⁵⁸ or release of chemoattractant from microspheres⁵⁹. However, many of these methods generate either poorly controlled gradients and/or gradients that change over time. Microfluidic platforms have emerged as controllable microdevices to be used in chemotaxis studies⁶⁰. Microfluidic systems allow the control and analysis of fluid dynamics at the micrometer scale⁶¹. The flexibility of these chambers has allowed the study of multiple soluble factors on different cell types⁶², to perform assays in 3D gels⁶³, chemotaxis essays under coculture conditions⁶⁴ and the generation of controlled nonlinear gradients⁶⁵.

Galvanotaxis (Electrotaxis):

Galvanotaxis (electrotaxis) is the phenomenon by which the movement of cells is directed in response to an electrical potential gradient. Studies in this field have allowed for characterizing the migrational behavior of different cell types in response to electric fields. Many cells migrate toward the cathode; however some migrate toward the anode. This migration direction can be altered depending on the expression of certain proteins⁶⁶. Most cells seem to migrate in gradients as high as 200 mV/mm, mimicking the *in vivo* range of 42-100 mV/mm across a wound bed, but can sense as low as 10-25 mV/mm⁶⁷. Speed on the other hand seems to be cell type and ECM dependent resulting in some studies showing no change in speed⁶⁸ and others showing either a biphasic dependence⁶⁹ or increasing speed with increasing electric field strength^{67a}. There is also some recent evidence that suggests that AC electric fields can augment DC electric fields and induce changes in directionality and speed⁶⁸. In general, the response direction and the threshold for migration varies across species and cell types⁷⁰.

The techniques to generate electric fields experimentally are varied, but rely primarily on generating them in chambers^{69,71}. These chambers have been designed to allow for the control over different electric fields^{71c} and in 3D matrices^{71d}. In addition, there has been some recent work developing chambers that can induce both electric field gradients and chemical gradients^{69,71a}, which is interesting given the use of overlapping signaling pathways during galvanotaxis and chemotaxis⁷².

Quantifying Random and Directed Cell Migration:

In order to quantify directed cell migration, parameters that describe the extracellular cue and the cell migration behavior must be made. Common approaches for measures are outlined in Table 1, however the literature is saturated with various ways in which to quantify directed migration.

Many of the approaches include calculating the average angle between the displacement of the cell and the direction of the cue (Figure 2). A less optimal approach is to replace the vector that describes the displacement of the cell with the vector that describes the long axis of the cell. This angle can be used in the calculation of tactic indices or directionality indices and can be averaged over several cells. In addition, related continuum transport parameters such as the chemotactic coefficient, χ or the drift velocity (Table 1). Often times the average value of the directional cue (fiber density, soluble molecule concentration, etc.) as well as the steepness of the gradient (or in the case of contact guidance, degree of alignment with can be quantified by the width of the angle distribution) set the efficiency of migration as defined by parameters such as the tactic indices. If the parameters that describe the directional cue change over time, the tactic and directionality indices more formally are functions of the average value and gradient steepness of the directional cue at previous times as well. Exposure to certain environments might result in either sensitization/amplification⁷³ or desensitization/adaptation⁷⁴ to certain extracellular cues, resulting in an enhanced or diminished response to the new environment. Indeed, in the context of chemotaxis, amplification and adaptation are common phenomena that drive current models⁷⁵. The influence of the previous exposure of cells to different environments could be described as cell memory and may partially decouple instantaneous cell migration responses to the environment in which the cell finds itself. However, most often there is an assumption that the tactic or directionality indices are in dynamic equilibrium with the input average values and gradient steepness. This has not been systematically examined, however studies showing how cells under certain circumstances “overshoot” concentration maxima and migrate down gradients might indicate memory⁶⁵.

The richness in directed cell migration behavior coupled with the ability to properly quantify it has resulted in numerous modeling efforts to explain directed cell migration in single cue environments. Some of these models have directly tied environmental inputs to directional migration outputs, effectively bypassing intracellular details^{27b, 76}. These models have taken the form of discrete single cell models or continuum transport models that may also incorporate remodeling of the environment, such as secretion or clearance of a chemoattractant or degradation and assembly of the ECM. To complement these efforts some have focused solely on intracellular signaling^{54a, 77} or cytoskeleton dynamics⁷⁸ using mass action kinetic, mechanical or phenomenological models. This is particularly evident in the chemotaxis field^{4c}, where numerous groups have tried to explain the necessity and genesis of adaptation and amplification in signal transduction that allows some cells to sense very shallow gradients. Consequently, several interesting models for contact guidance^{27b, 76a}, chemotaxis^{4c}, haptotaxis⁷⁹, galvanotaxis⁸⁰ and durotaxis⁸¹ have emerged.

Now that random and directed migration, platforms to control the spatial organization of directional cues and important quantitative variables that describe both random and directed migration have been introduced, we will discuss the smaller, but mounting number of studies conducted examining multi-cue directional migration. We write about these studies in the context of five general behaviors that have been seen to date. We will finish by examining environmental factors that might switch the dominance of directional cues as well as experimental, analysis and modeling challenges associated with understanding directed migration in complex multi-cue environments.

Multiple Cues in Cell Migration:

As mentioned above directional cues are often simultaneously presented. For instance, during wound healing electric fields and chemoattractant gradients of growth factors may drive epidermal or fibroblast cell migration into the provisional clot^{72, 82}. Multiple chemoattractants such as cytokines and fMLP secreted by non-immune cells in response to inflammation and by invading bacteria may form gradients that direct immune cell migration to the infection site¹¹. Finally, chemoattractant gradients of growth factors and aligned fields of collagen fibers drive cancer cell migration out of the primary tumor^{28a}. There is some very nice work trying to understand how multiple uniformly distributed cues quantitatively regulate random cell migration. For instance, cell speed, membrane extension/retraction activity and adhesion were analyzed on different fibronectin densities and different EGF concentrations^{25b}. EGF can change the point of maximal speed by reducing the strength of cell-substratum adhesiveness. In addition, changing the substrate stiffness keeping the collagen concentration the same also resulted in a shifting of the maximum⁸³. However, inhomogeneous cues elicit directed responses and as such the relative orientation of the cues is also important leading to situations where different cues can either cooperate with or compete against each other. How might this occur?

The relative orientation of the directional cues will determine whether cooperation or competition will occur (Figure 1B). For monodirectional cues such as those that regulate haptotaxis, durotaxis, chemotaxis and electrotaxis, cooperation occurs when the angle is 0° and competition occurs when the angle is 180° . For bidirectional cues such as those that regulate contact guidance cooperation occurs when the angle is 0° and competition occurs when the angle is 90° . Intermediate angles result in some intermediate cooperation/competition scenario. Once competition or cooperation is established, there are several factors that drive which cues dominate and which submit. First, the average value of the cue such as the average concentration

or stiffness determines whether the cell migrates at all. For instance, if the ECM concentration or stiffness is too high or too low, the cell does not migrate because it adheres tightly to the substrate or generates too small of a traction force to pull itself forward. Additionally, if the soluble factor concentration is too high or too low, a gradient in receptor occupancy across the cell is not perceived. Receptors will either be saturated at both the front and rear of the cell or the concentration will be too low and noise associated with the number of receptors occupied at any one time will swamp differences between receptor occupancy between the front and the rear. Directional cues at suboptimal average values are likely to submit to cues at optimal average values. Second, the intensity of the cue such as the degree of alignment or gradient steepness determines the degree to which migration is biased. If the degree of alignment or gradient steepness is too low, the cell will not sense the directional cue. Directional cues at low degree of alignment or gradient steepness are also likely to submit. How does this integration of multiple pieces of information affect the directionality of the cell? Several scenarios could exist. The first is a simple vector addition of the migrational response. When cues are aligned, this might produce an additive effect of the cell migration speed and/or directionality. For instance, if cue 1 and cue 2 each generate a directionality of 0.4, the combined effect would be a directionality of 0.8 (cooperation) when oriented at 0° and 0 (competition) when oriented at 180° . Assuming that the directional response to each cue is not saturated, one might be able to enhance the ability of cue 1 to cooperate or compete by increasing degree of alignment or gradient steepness of cue 1. However, simple additive responses may not always govern multi-cue migration. Below are five examples of behavior that have been identified in multi-cue directional migration systems.

Immunological Chemotactic Cues- Hierarchical Dominance and Cooperative Relay

Systems:

Multiple chemotactic cues have been assessed primarily in the context of immune function. Using the under agarose assay, the influence of overlapping chemotactic gradients on the migrational behavior of neutrophils was evaluated⁸⁴. This data demonstrated that when opposing gradients of chemoattractants of IL-8 and LTB4 are present, cells selected the direction of migration based on the midpoint concentration and gradient steepness and no one chemoattractant dominated. If the gradient's midpoint concentration of a particular cue was too high, receptors were saturated at both the front and rear of the cell, eliminating any ability to sense that gradient. The authors then described this as a preference for distant sources of chemoattractant. However, the integration of these signals is most likely not additive as other experiments in more controlled gradients show LTB4 is better at competing than IL-8⁸⁵. Other chemoattractants such as fMLP or C5 α when presented with either IL-8 or LTB4 dominated the migrational behavior, even at high gradient midpoint concentration, most likely due to distinct signaling pathways⁸⁶. This hierarchical domination may be important physiologically as fMLP and C5 α are related to finding and killing foreign invaders, whereas IL-8 and LTB4 are related to inflammation. Hierarchies in chemotaxis have also been found elsewhere . Finally, there is work demonstrating interesting cooperation between chemoattractants. Given a source of IL-8 or LTB4 alone that is above the saturation concentration, cells will not fully migrate to the source. The bias in migration is eliminated at a distance where the front and rear receptors (or intracellular signaling pathways) are saturated. However, if another chemoattractant is present, it can take over the role of biasing migration after saturation of the first chemoattractant sensing system. This sequential sensing of chemoattractants may be a mechanism by which cells migrate

to targets over a tremendous length, essentially setting up a chemotactic relay system, where one chemoattractant directs migration before handing the role off to another chemoattractant.

Whether during cooperation or competition it is tempting to suggest the existence of cell migration targets, which are spatially distinct from chemoattractant sources and can be determined by the gradient magnitude and midpoint concentration of multiple cues⁸⁷. In this situation, desensitization of signaling might produce nonintuitive results such as migration down gradients of chemoattractant⁸⁸. In one report T-cells did not migrate in CCL19 gradients unless in the presence of CCL21 and then only towards lower concentrations of CCL19. This was explained by a mathematical model of CCR7 desensitization, where one chemoattractant was more potent in desensitizing the response⁸⁹. A similar response was found in dendritic cells⁹⁰.

The majority of the controlled gradients discussed above were created in microfluidic chambers and all, with the exception of one study^{90a}, were conducted using cells migrating in 2D. The platform of microfluidics will continue to be an important technique to control multiple chemotactic cues. However, most of the microfluidic chambers used so far only have the ability to present cues in parallel fashion. However, new microfluidic designs have opened up possibilities to organize several spatial gradients in interesting ways⁹¹. This gives microfluidics a better opportunity to compete with micropipettes or loaded beads, where multiple gradients are perhaps more easily generated even though the gradients are less controlled. In addition, controlling multiple gradients in 3D environments will more likely mimic multi-cue sensing *in vivo*. Finally, what is lacking from most of these studies is the correlation between gradients in close proximity to the cell. Many of these studies simply performed analysis on groups of cells that are not exposed to the same gradient characteristics. In addition, the assumption that the migration characteristics are in dynamic equilibrium with the environmental conditions at each

time might be a poor assumption. Memory caused by either sensitizing or desensitizing signal transduction networks needs to be examined more closely in multi-cue environments. This might be particularly important in situations where different cues use overlapping signal transduction networks, perhaps leading one cue to prime or turn down the response to another cue.

Multiple Haptotactic and Chemotactic Cues- Cooperation Can Overcome a Threshold and Immobilization Matters

The combined action of haptotactic and chemotactic signals has also been investigated in the context of multi-cue directed cell migration. To evaluate the cumulative guidance effect of NT-3 and NGF, single and superimposed soluble concentration gradients were formed in under agarose assays ⁹². The primary receptors for NT-3 and NGF are distinct, but co-localized. Consequently, separate signaling pathways could be elicited ⁹³. A threshold gradient of NGF was required for neurite guidance, a closely related process to cell migration. Interestingly, when dual concentration gradients of NT-3 and NGF were constructed with each individual gradient below the threshold gradient for NGF, directional extension of neurites occurred. Consequently, dual gradients can overcome threshold constraints for directed extension. In addition, directed extension occurred when gradients of immobilized NT-3 and NGF were formed on p(HEMA) substrates, albeit at steeper gradients ⁹⁴. These differences open the possibility that the steering mechanisms of neurites and cells depend on the way that cues are presented, and this could affect how cues compete in multi-cue environments. Indeed this has been shown in leukocytes. Chemotaxis and haptotaxis experiments were performed in a modified Boyden chamber ⁹⁵. Human growth hormone (hGH) promoted directional movement through both chemotaxis and haptotaxis alone. When RANTES gradients were overlaid with hGH gradients, an abolishment of

the migration occurred, but this only occurred if the hGH chemotactic gradients were used in combination with RANTES chemotactic gradients or hGH haptotactic gradients were used in combination with RANTES haptotactic gradients. If hGH haptotactic gradients were used in combination with RANTES chemotactic gradients, or vice versa, migration was not inhibited. This result highlights that the presentation of the ligand as either a chemotactic or haptotactic gradient can determine the dominance of a directional cue over another. In addition to immobilization, the midpoint concentration of one of the attractants can also dramatically impact the behavior of directed migration. Studies carried out using a novel microfluidic platform that can be used to make multiple gradients oriented in the same or opposite configuration demonstrated that the ability of BDNF to repel growth cone extension in a haptotactic gradient of laminin that depended on the steepness and/or the midpoint concentration of the BDNF gradient⁹⁶.

Most of the above tactic gradients were created on or above 2D glass surfaces and take advantage of microfluidic chambers although several other techniques exist to make controlled multidimensional haptotactic gradients⁹⁷. However, generating gradients on other surfaces is important as well. More recently, different arrangements of overlapping gradients of multiple proteins were immobilized on PEG hydrogels using microfluidic platforms⁹⁸. With this technique, linear and non-linear gradients can be generated, but the effective sequential immobilization of proteins using this methodology is precluded to a certain extent due to the lack of free binding sites when parallel gradients are generated. Nevertheless, the combination of two orthogonal binding schemes allows the independent tethering of two proteins on a hydrogel surface. In addition, the ability to generate different gradients at different z positions in a 3D matrix will be important in building 3D multi-cue environments. A newly developed benchtop

technique based on capillary flow and molecular diffusion to generate concentration gradients has been reported. Multi-gradient hydrogels are fabricated layer by layer using an open channel and subsequent crosslinking of the gradient precursor⁹⁹. In addition, photochemical techniques that allow for attachment of haptotactic signals to 3D matrices show promise¹⁰⁰. Although these studies show important advances in terms of methodologies to generate haptotactic gradients on physiologically relevant substrates, some concern in regards to the stability of the gradients within time and the bioavailability of the molecules depending on the tethering mechanism are still being assessed. Unfortunately, the use of such platforms to address cell migration behavior with parallel or competing gradients has not been reported.

Galvanotaxis and Chemotaxis: Evenly Matched

As describe above, galvanotaxis constitutes a complex process that depends strongly on cell type and the specifics of electric field induced. Interest in galvanotaxis and its biomedical applications is continuing to build. A first attempt to study the interaction of chemotaxis and galvanotaxis was recently reported¹⁰¹. Radio frequency electric currents were generated in parallel to gradients of the chemoattractant, cAMP and directed migration of human neutrophils was reported. Once the electric field was imposed, neutrophil speed increased about 50%, and more importantly, changed direction to align perpendicularly to the alternating electric field. These results suggest that alternating electric fields may dominate chemotaxis. In another study, T cells moved preferentially towards the cathode that induced a direct current electric field. They also showed migration of T cells towards high concentrations of CCL19 gradients. When both were applied in opposing fashion, the orientation of the cells was essentially random, indicating that the electrotactic gradient can compete well with the gradient in CCL19⁶⁹. In this context the

reduction in orientation index suggests that the interaction between these two directional signals is the sum since each level alone is roughly the same.

Devices to examine galvanotaxis in combination with other directed migration mechanisms has the added difficulty of fabricating functional electric circuits. In the above study, Li et al. performed experiments in a microfluidic device designed to present coexisting chemical gradients and direct current electric fields⁶⁹ and specific challenges associated with these devices have been outlined previously^{71a}. In terms of design, this platform allows one to superimpose and independently control the two types of signals in time-varying electric fields, as are sometimes presented during wound healing.

Haptotaxis and Durotaxis: Chemical Signals Lead the Way

Several studies have revealed the effects of both stiffness gradients and haptotactic gradients in controlling cell movement, but it is still unclear which dominates. In a recent study, the motility of cells under the influence of both mechanical and chemical signals was described¹⁰². Polyacrylamide gels drops with different concentrations of crosslinker and different concentrations of collagen were merged generating opposing gradients in collagen concentration and Young's modulus. During durotaxis, cells migrate towards regions of higher Young's modulus. Additionally, cells migration up gradients of collagen. Hale et al. showed that 3T3 fibroblasts migrated preferentially towards the high collagen and low modulus regions of the substrate. Additionally, when the gradient of collagen decreased and the gradient of stiffness increased, the amount of cells migrating in that direction decreased. Unfortunately, this study did not examine systematically the steepness or midpoint concentration of either the Young's modulus or the collagen coverage. In addition the collagen concentration was only very roughly

estimated. However, these preliminary data indicate that chemical cues can win out over mechanical cues causing cells to migrate down a gradient of mechanical stiffness.

Polyacrylamide (PAAM) hydrogels are optimal substrates for tuning mechanical properties. Other polymers might be appropriate as well, however, recent work has indicated that while bulk stiffness can direct cell behavior, crosslinking and ECM flexibility is an important factor. Polymers like PDMS can show dramatically different cell behavior even when the bulk modulus is the same¹⁰³. The above technique is an interesting way in which to generate mechanical stiffness gradients, but the generation of the collagen gradient is much less controlled. In addition to PAAM hydrogels, gradients in mechanical stiffness can be generated by compression of collagen gels¹⁰⁴. However, because collagen concentration regulates mechanical stiffness in these gels, parsing durotactic and haptotactic effects is challenging. Photochemical techniques can also be used to generate gradients in stiffness¹⁰⁵ as well as crosslinking⁵¹ and might generate a better spatially controlled environment.

Contact Guidance and Chemotaxis: Cell Type Specific Dominance

An immune cell's response relies strongly on chemotaxis induced by soluble factors at the infection site that direct its movement. In addition, as the wound matures, fibroblasts align fibers in the ECM. Under this premise, one of the first studies in which different signals were presented simultaneously in a migration assay was conducted¹⁰⁶. Neutrophil chemotactic migration towards candida was only somewhat stunted if the chemotactic and contact guidance signals were presented perpendicularly. The same result was seen in gradients of fMLP, indicating that in immune cells chemotaxis dominates. Chemotaxis was seen to also highly influence the orientation of fibroblasts in fibrin microspheres¹⁰⁷. Fibroblasts seeded in the shell of concentric

spheres contract fibrin matrices forming circumferential fibers. This alignment then causes fibroblast alignment and contact guidance. Inducing radial chemotactic gradients by embedding macrophages that secrete chemoattractants in the core, effectively abolished the circumferential alignment and contact guidance, suggesting that like neutrophils, chemotaxis can dominate contact guidance. However, this result is convoluted due to significant change in the contact guidance cue over time. The fibrin gels initially were randomly organized and the aligned fibrin matrix only developed over time. In addition, contact guidance was better able to compete and dominate chemotaxis at longer times after the directional cue of the fibrin fiber alignment developed. The final and most recent examination of the competition between chemotaxis and contact guidance was in HUVECs migrating on electrospun fibers and in gradients of VEGF¹⁰⁸. This tentatively suggests that chemotaxis might dominate in less contractile cells (neutrophils), whereas contact guidance might dominate in more contractile cells (fibroblasts and HUVECs), setting up the contractile state of the cell as a regulator of which directional cues dominate and which submit.

Although both chemotactic and contact guidance signals were presented simultaneously in space and time Lackie et al. did not quantify chemoattractant gradients or systemically examine different chemoattractant and contact guidance environments. In addition, Bromberek et al. did not quantify or control the chemotactic gradient. The most promising platform was that used by Sundararaghavan et al., where electrospinning and microfluidic chambers were combined. In addition to electrospinning, microcontact printing lines¹⁰⁹ or other approaches may be used in combination with microcontact printing lines. However, these still constitute 2D environments in which to monitor cell migration. Consequently, microfluidic chambers in which

matrices such as collagen gels can be assembled¹¹⁰ and aligned^{142a, b} might be the best approach to examining the competition between contact guidance and chemotaxis.

Switching the Dominance of Directional Cues:

As discussed in the previous section, it is tempting to speculate that the contractile state of the cell mediates the dominance of either contact guidance or chemotaxis. In addition to contractile state, proteinase activity might also play a role in switching the dominance. As mentioned briefly above, two major modes of individual migration have been characterized: amoeboid and mesenchymal. Amoeboid migration is proteinase independent and is less reliant on interactions with the ECM, which might explain why most chemotaxis systems have been described as amoeboid. Mesenchymal migration is proteinase dependent and is more reliant on interactions with the ECM, which might suggest a stronger contact guidance response. Since researchers have shown switching between amoeboid and mesenchymal migration¹¹¹, perhaps dominance of chemotaxis (amoeboid migration) or contact guidance (mesenchymal migration) could be switched as well. Other perturbations at the level of signal transduction or cytoskeleton regulation might also act similarly. Recently, there have been a couple of examples of molecules or pathways that affect only a certain type of taxis. For instance, chemotaxis and haptotaxis use different cytoskeleton components¹¹². Wu et al. demonstrated that Arp2/3 is only active in haptotaxis and blocking its action does not affect chemotaxis. In addition, force feedback loops that generate oscillations in traction force at focal adhesions seem only to be important during durotaxis as compared to chemotaxis or haptotaxis¹¹³. Augmenting either Arp2/3 or force oscillations at focal adhesions might allow for the switching in dominance between competing cues and might constitute a mechanism to redirect migration in complex environments.

Final Remarks:

Throughout this review we have discussed general principles for random migration and directed migration in response to inhomogeneous chemical, mechanical or electrical cues. We have described how one can quantify important characteristics of the spatial inhomogeneity of the cue as well as the bias in cell migration direction. We have also described approaches to control spatially inhomogeneous cues and study directed cell migration *in vitro*. Most importantly we have reviewed the roughly twenty studies examining multi-cue directed migration. Striking qualitative behaviors have been found like hierarchical dominance, cooperative relay systems, cooperative threshold alteration and cell-type specific dominance. However, several challenges remain.

The first challenge refers to the design and fabrication of multi-cue environments to control the spatial distribution of several directional cues. Advancements in microfabrication and materials science have provided tools to meet these challenges (Figure 3). In particular, microfluidics have pushed the ability to control soluble and surface bound directional cues and can be combined with other approaches to control electric fields ^{71a} and aligned fibers or fiber-like materials. However, other controlled release mechanisms for soluble gradient control will nicely complement these approaches, particularly in tissue engineering contexts. More techniques are needed to generate controlled properties of aligned fibers and gradients in mechanical properties. This is challenging given that fiber organization and mechanical properties tend to be linked. In addition, bulk mechanical properties might not properly describe the local mechanical environment of cells ¹¹⁴, necessitating local mechanical measurements of properties and gradients in properties. Finally, multi-cue environments that display the same

topology, confinement and mechanical properties as 3D systems will be most informative. Most of the studies to this point have been performed in 2D environments, but for good reason. One yields much less control over the spatial distribution of cues in 3D environments. In addition, imaging subcellular dynamics in 3D is much more challenging than in 2D. Perhaps hybrid environments that retain certain features of 3D migration such as topology, confinement or mechanical properties are a reasonable compromise¹¹⁵. While most of these studies have examined directed migration behavior only, environments in which subcellular dynamics of signaling and the cytoskeleton will be needed in order to form mechanisms for cooperation, competition, dominance and submission. Finally, in order for specific environments to make a large impact, they must be made accessible to non-specialists. Fee-for-service nano- and micro-foundries have perhaps loosened the ease-of-construction constraint, particularly for the specific case of microfluidic devices. However, the ease-of-use constraint is still critical, given non-specialists must populate the environments with cells and make specific measurements related to migration.

These technological advances will need to be met with sophisticated and thorough quantitative analysis and this constitutes a second challenge. Quantitative analysis of the cell migration behavior will be needed to determine thresholds, assess additive versus synergistic mechanisms and to fully uncover the degree of dominance of one cue over another. Quantitative analysis of the signaling and cytoskeleton dynamics will be needed to assign molecular mechanisms to competition, cooperation, dominance and submission. In addition, a unified set of quantitative metrics might allow for the comparison of multi-cue directional sensing in several different systems in search for governing principles. Finally, mathematical modeling holds a place in predicting thresholds, assessing molecular mechanisms for synergy and explaining dominance by certain cues over other cues. As mentioned above models of intracellular

processes have been used extensively in the spatial sensing/chemotaxis field to understand how amplification, adaptation and other processes allow the cell to transmit the external gradient into a gradient in signaling and cytoskeleton assembly. However, only a few models have been developed for multi-cue sensing^{89, 116}, but show promise in explaining why cues compete to direct cell migration. In addition, data-driven models that have extended the understanding of the response to multiple inputs in signal transduction cascades will be useful tools for multi-cue directional sensing¹¹⁷. What is really exciting is the possibility that models of intracellular processes like signal transduction and cytoskeleton dynamics could be incorporated into higher level models that describe cell migration in a population of cells in order to predict tissue dynamics in diagnostic samples¹¹⁸ or tissue engineered constructs¹¹⁹.

We are just beginning to understand how cells integrate multiple directional cues during migration. This integration is critically important given that cells are exposed to complex multicomponent environments in vivo. Understanding how multiple cues impact cell migration will advance cancer diagnostics by allowing cancer cell migrational behavior to be predicted in biopsies where the distribution of multiple migrational cues can be measured or tissue engineering by informing the design of constructs that orchestrate the assembly of tissue through presentation of cues that directed where and how fast different cell types migrate toward their final targets.

Tables:

Table 1. Quantitative parameters that describe extracellular cues and cell migration.

Type of Migration	Cue parameters	Cell Parameters
Random	C_{ECM} [=] $\mu\text{mol}/\mu\text{m}^2$: ECM concentration	$\langle d^2(t) \rangle = n_d S^2 P \left(t - P \left(1 - e^{-(t/P)} \right) \right)$
	C_{sol} [=] μM : soluble factor concentration	d [=] μm , displacement
	E : Young's Modulus	n_d [=] 1, number of dimensions
		S [=] $\mu\text{m}/\text{min}$, migration speed
		P [=] min, persistence time
		$D = \frac{S^2 P}{n_d}$
		D [=] $\mu\text{m}^2/\text{min}$, motility coefficient
		f [=] s^{-1} , tumbling frequency
		t_{run} [=] s, run duration
		θ_{random} [=] rad, turn angle distribution
	$MI = \frac{d(t)}{St} = \sqrt{\frac{n_d P}{t}}, t \gg P$	
	MI [=] 1, migration index	
Contact Guidance	ρ [=] $\#/\mu\text{m}^2$ or $\#/\mu\text{m}^3$, fiber density	$DI = \langle \cos 2\phi \rangle$
	σ_{fiber}^2 [=] $(\#/\mu\text{m}^2)^2$ or $(\#/\mu\text{m}^3)^2$, degree of alignment or a parameter that describes fiber angle distribution like standard deviation	DI [=] 1, directionality index
	d_{fiber} [=] μm , fiber diameter	$D_A = \frac{D_x}{D_y}$
	Δn [=] 1, birefringence	D_A [=] 1, anisotropic motility coefficient

		D_x and D_y [=] $\mu\text{m}^2/\text{min}$, motility coefficient in a particular direction
		MI_x and MI_y [=] 1, migration index in a particular direction
Chemotaxis	$\langle C \rangle$ [=] μM , mean solute concentration	$CI = \langle \cos \phi \rangle$
	∇C [=] $\mu\text{M}/\mu\text{m}$, gradient steepness	CI [=] 1, chemotactic index
		χ [=] $\mu\text{m}^2/\text{min } \mu\text{M}$, chemotactic coefficient
		$CP = e^{\sin 2\phi}$
		CP [=] 1, compass parameter
Haptotaxis	$\langle \Gamma \rangle$ [=] μM , mean ECM concentration	$HI = \langle \cos \phi \rangle$
	$\nabla \Gamma$ [=] $\mu\text{M}/\mu\text{m}$, gradient steepness	HI [=] 1, haptotactic index
		S_h [=] $\mu\text{m}/\text{min}$, drift velocity
Galvanotaxis	$\langle E \rangle$ [=] μM , mean electric field strength	$EI = \langle \cos \phi \rangle$
	∇E [=] $\mu\text{M}/\mu\text{m}$, gradient steepness	EI [=] 1, electrostatic index
		S_e [=] $\mu\text{m}/\text{min}$, drift velocity
Durotaxis	$\langle G \rangle$ [=] μM , mean stiffness	$EI = \langle \cos \phi \rangle$
	∇G [=] $\mu\text{M}/\mu\text{m}$, gradient steepness	EI [=] 1, durostatic index
		S_d [=] $\mu\text{m}/\text{min}$, drift velocity

Table 2: Synopsis of reports of multiple directional cues

Cue Combination	Competition	Dominant Cue	Cooperation	Platforms	Cells	Year	Citation
contact-chemo	n/a	n/a	yes	fibrin gels	leukocytes	1983	¹⁰⁶
hapto-chemo	n/a	n/a	yes	Boyden	leukocyte	1995	⁹⁵
chemo-chemo	yes	fMLP and C5a > IL-8 and LTB4	yes	under agarose	neutrophil	1997	⁸⁴
contact-chemo	yes	chemo > contact	n/a	3D spherical gel	fibroblast	2002	¹⁰⁷
chemo-chemo	yes	fMLP and C5a > IL-8 and LTB4	n/a	under agarose	neutrophil	2002	⁸⁶
chemo-chemo	yes	IL-8 > LTB4	n/a	microfluidic	neutrophil	2005	⁸⁵
hapto-hapto	yes	NGF > NT-3	n/a	gradient maker TM	DRG cell	2006	⁹⁴
hapto-hapto	yes	BDNF > laminin	n/a	microfluidic	neuron	2008	⁹⁶
galvo-chemo	yes	galvo > C-AMP	n/a	strip source diffusion	neutrophil	2008	¹⁰¹
hapto-chemo	n/a	n/a	yes	SAMs/diffusion	hMEC	2009	¹²⁰
chemo-chemo	yes	CCL19 > CCL21 and CXCL12	n/a	microfluidic	dendritic cell	2010	⁸⁷
duro-hapto	yes	collagen > duro	n/a	PAAM hydrogels	fibroblast	2010	¹⁰²
chemo-chemo	yes	CCL21 > CCL19	n/a	microfluidic/agarose	dendritic cell	2011	^{90a}
chemo-chemo	yes	CCL21 > CCL19	n/a	microfluidic	T cell	2011	⁸⁸

hapto-hapto	n/a	n/a	yes	microfluidic	DRG cell	2011	121
galvo-chemo	yes	galvo > CCL19	n/a	microfluidic	T cell	2012	69
chemo-chemo	yes	fMLP > CXCL8 > CXCL2 > LTB4	n/a	microfluidic	neutrophil	2012	90b
contact –chemo	yes	contact > VEGF	yes	Microfluidic /electrospinning	HUVEC	2013	108

Figure Legends:

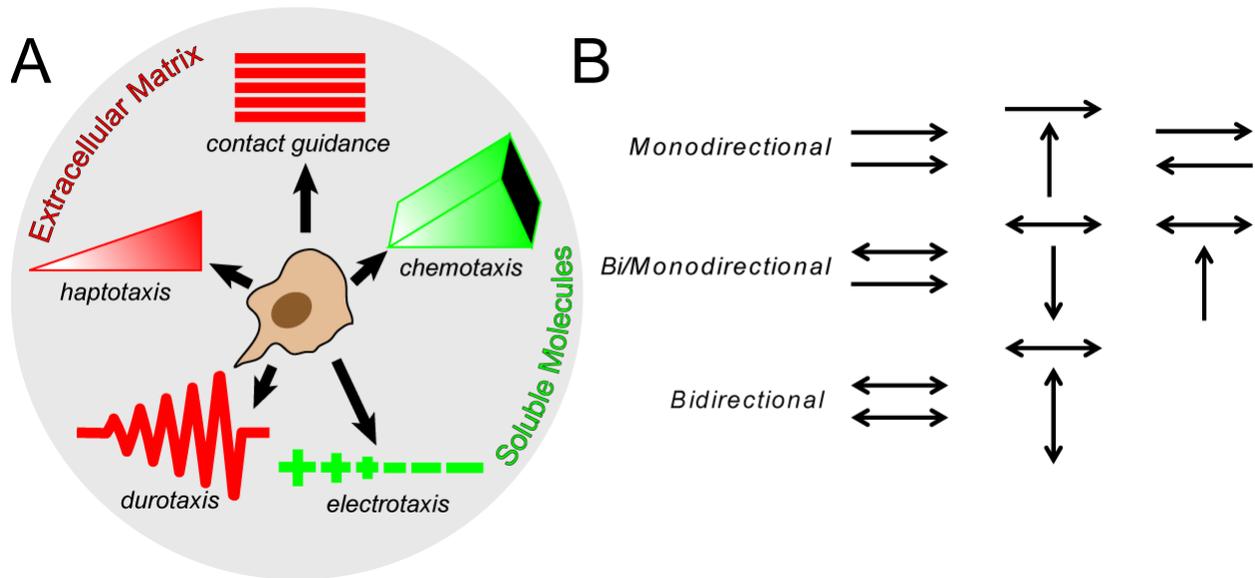


Figure 1: Diversity in Directed Cell Migration and Ways in which Directional Cues Can

Either Cooperate or Compete. A. Cell migration can be directed either by ECM, soluble factors or electric field transmitted by ion flow. These directional cues can either be monodirectional or bidirectional and bias migration based on either alignment of fibers or gradients in ECM density, stiffness, soluble factor concentration or electric field. B. While contact guidance is bidirectional, all other forms of taxis are monodirectional. In addition, these cues can be oriented differently to either cooperate or compete.

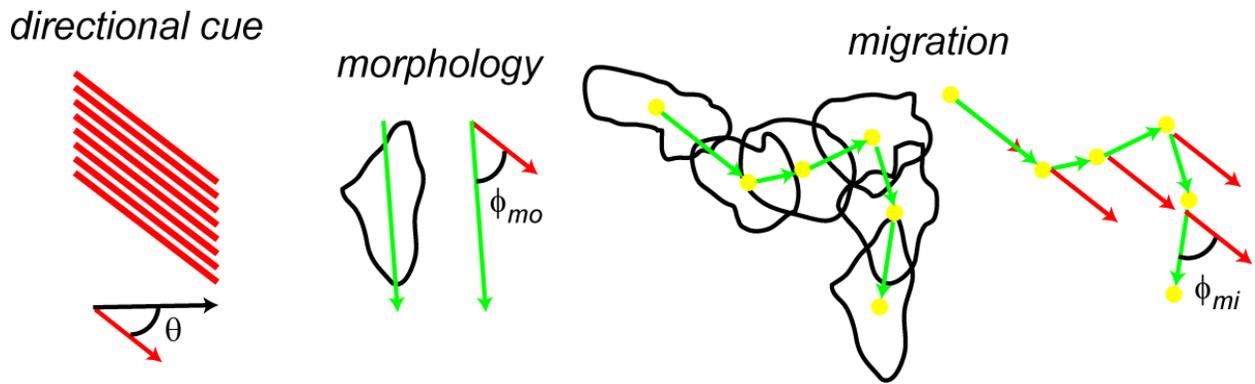


Figure 2: Quantifying Directional Migration. Directional cues such as gradients or aligned fibers can be characterized with an angle, θ that denotes the angle between the cue direction and a reference direction. For fibers that are not perfectly aligned $\theta = \langle \theta_i \rangle$, where $\langle \theta_i \rangle$ is the average angle between the direction of each fiber and a reference direction. The vector along the long axis of the cell can be used to assess how much influence the directional cue has on migration by calculating ϕ_{mo} . The better measure uses the vector that describes the displacement to calculate ϕ_{mi} . Both ϕ_{mo} and ϕ_{mi} can be used to calculate tactic or directional indexes using either $\cos(\phi_{mo})$ or $\cos(2\phi_{mo})$, respectively.

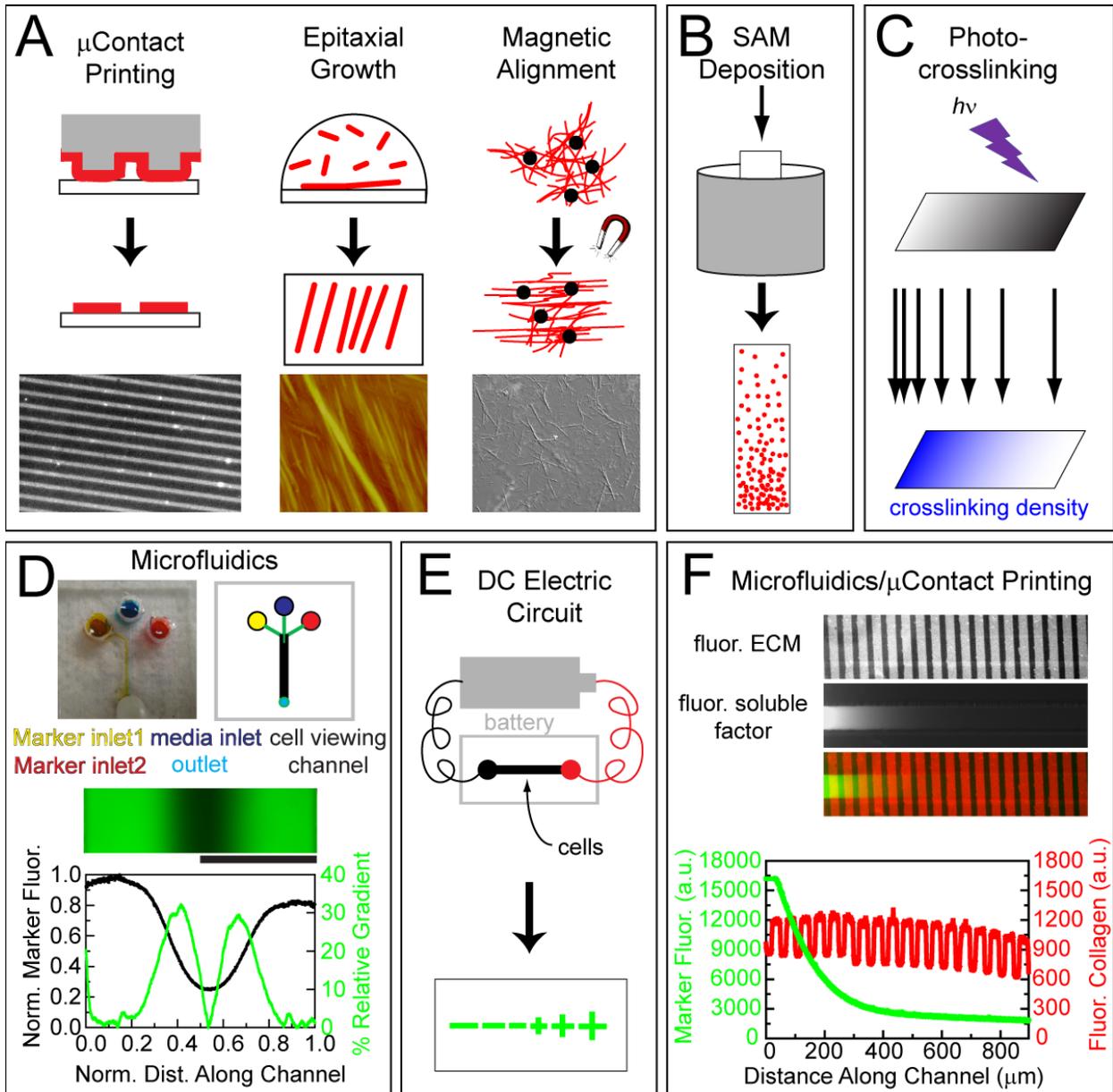


Figure 3: Schematics of Processes or Devices to Spatially Organize Migrational Cues. A. Contact guidance cues can be organized in 2D via microcontact printing or epitaxial growth on mica and in 3D via magnetic bead alignment. B. Haptotactic gradients can be produced by pulling gold coated substrates through solutions of functionalized thiols, creating SAMs with gradients in functional groups. C. Polyacrylamide/bisacrylamide polymer solutions can be selectively crosslinked by replacing the usual initiator with a UV-sensitive initiator, generating a

gradient in polymerization and crosslinking density that results in a gradient in mechanical properties. D. Chemoattractants can be fed through inlets in microfluidic chambers to generate gradients in soluble molecule. Cells are plated in the long, viewing channel. E. Chambers similar to those used as microfluidic chambers can be fitted with electrodes connected to a DC power source creating an electric field across the cell viewing chamber. F. Microcontact printing and microfluidics can be combined to generate orthogonal directional cues.

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