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Gradient generation platforms: new directions for an established microfluidic technology

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Microscale platforms are enabling for cell-based studies as they allow the recapitulation of physiological conditions such as extracellular matrix (ECM) configurations and soluble factors interactions. Gradient generation platforms have been one of the few applications of microfluidics that have begun to be translated to biological laboratories and may become a new "gold standard". Though gradient generation platforms are now established, their full potential has not yet been realized. Here, we will provide our perspective on milestones achieved in the development of gradient generation and cell migration platforms, as well as emerging directions such as using cell migration as a diagnostic readout and attaining mechanistic information from cell migration models.

Introduction

One of the dreams of microscale cell-based in vitro modeling is the recapitulation of cell signaling and tissue organization occurring in vivo in order to develop more physiologically relevant and/or higher throughput research platforms[1-3]. In this context, gradients are ubiquitous as signals secreted by cells diffuse into the extracellular matrix until they are cleared by flows from vessels, or degraded by enzymes. Numerous cell processes have evolved to recognize the directional information encoded in gradients, including many developmental processes such as neuron guidance[4], recruitment of immune cells (most often referred to in the field as chemotaxis)[5], angiogenesis[6], and diseases such as cancer[7].

While many microscale cell-based platforms are still in developmental and demonstration stages, micro scale gradient generation has begun to find more wide spread use[8]. The rising use of microfluidics in neutrophil and cell-migration platforms is fuelled by the limitations of traditional methods, particularly the lack of control over the gradient generation and the migration path[9]. Gradient generation platforms leverage one of the fundamental properties of fluids at small scales, namely the inherent ability to control diffusion over convection. These platforms enable the creation of gradients of soluble factors reliably and at unprecedented lengths and time scales. Here, we will provide our perspective on several key milestones in the field of microengineered gradient generation as well as important applications for these platforms. Finally, we will expand on exciting directions gradient-based in vitro platforms are taking and important technological opportunities that these platforms offer.

Gradient generation platforms

Examination of the properties of fluids at the microscale has led to the realization that the effects of inertia (leading to instabilities and turbulences) are relatively weak compared to other effects such as viscosity, surface tension, and diffusion[10]. These characteristics can be assessed using non-dimensional numbers such as the Reynolds number (viscosity vs. inertia), the Peclet number (convection vs. diffusion)[11], or the Bond number (gravity vs. surface tension). An important consequence is that diffusion - normally a very weak phenomenon - is the main driver of fluid mixing at the microscale[12]. The foundational principle for creating gradients is that two fluids with differing concentrations of a diffusible molecule will, through diffusion, generate a gradient as the higher concentration merges into the lower concentration[13]. As diffusion is a very predictable phenomenon, microscale platforms offer a high degree of control over the spatio-temporal fluidic patterns and allow the creation of gradients through many approaches[9]. An important effort has focused on generating gradients with controllable profiles, timescales, chemical natures, and in physiologically relevant matrices and tissue organizations. Currently, gradient generation platforms can be generally classified into five categories:

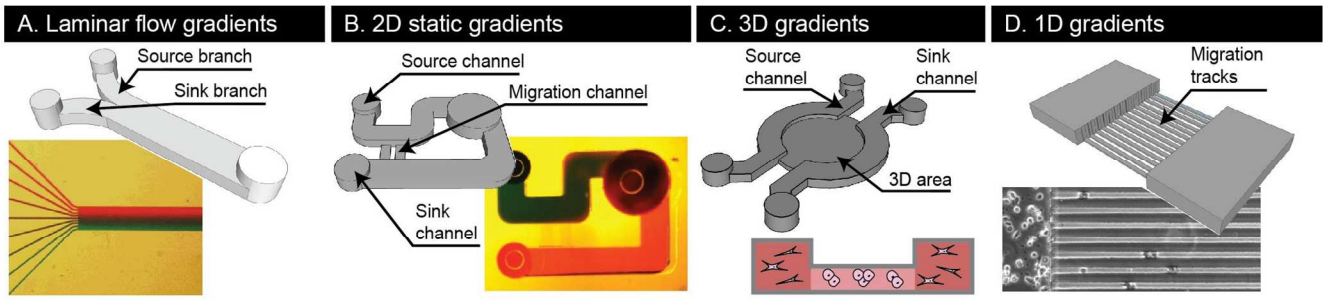

Figure 1: A. Laminar flow gradient generation; two or more branches of different concentrations merge into one channel in which the gradient is generated transversally to the direction of the flow. B. Schematic of a static gradient generation platform; two channels are connected by a thin channel that prevents fluid flow. Cells placed in one of the two channels (usually the sink) invade the connecting migration channel. C. Schematic of a 3D gradient device; a plug of hydrogel is placed (e.g. pinned) between two channels, generating a gradient in the 3D matrix. (D) Schematic of a 1D migration device; thin capillaries of general size of a cell connect 2 reservoirs. Migrating cells migrate into the thin capillaries leveraging the constricted environement.

Laminar flow gradients - The earliest and most widely used gradient generation platforms leverage laminar flow properties to flow two (or more) fluids of different compositions side by side in a channel (Figure 1A). This is typically established using a Y channel (or multiple Y inlet channel) in which fluids of different concentration flow in each branch of the Y. Diffusion forces causes a progressive mix of compounds contained in each fluid, creating a gradient transverse to the direction of the flow[14, 15]. These gradients have the advantage of being stable over time[16], can be formed in very short length scales down to the cellular level[17], and offer unrivaled precision in timescales[18, 19]. However laminar flow-based gradients contain a number of limitations. They are typically hard to multiplex due to the presence of tubing and connectors. The shear stress induced by the constant flow can affect cellular migration as well as induce undesired signaling events. Finally, maintaining a steady state gradient is complex and requires highly precise equipment[20]. For these reasons, the use of these type of gradients is diminishing.

Static gradients - Static gradient generation platforms have been developed to respond to the need for (1) higher-throughput gradient platforms, (2) reduced interference due to shear stresses, and (3) improved ease-of-use enabling their translation into biological laboratory settings[21] (Figure 1B). Typically these platforms integrate a strategy to prevent convective flows through the integration of sections with high fluidic resistance, such as a microporous membranes[5], gel walls[22], or thin aspect ratio channels[23]. As convection is not actively controlled, fluidic features to divert flows have been developed for increased robustness[23]. Further, the use of static gradients enables a broader range of geometries resulting in the ability to control the shape of the gradient and the development of non-linear gradients[24] and point-source gradients[25]. These gradients, in general, require a longer setup time and offer less control over the diffusion distance and dynamic properties of the gradient profile. The improved ease-of-use, however, has resulted in static gradients becoming more generally accepted in biology labs, a trend highlighted by the emergence of commercially available gradient platforms such as the BellBrook[26] or ibidi[27] platforms, which are becoming "gold standards" for cell migration studies.

3D gradients - The need for models with increased biological relevance due to improved understanding of the critical importance of the microenvironment has led to increased focus on gradients generated in 3D matrices (Figure 1C). Interestingly, while 3D microenvironments induce complexity in the development of in vitro models and readouts, they are typically more amenable to gradient formation due to the general lack of convection[28]. These properties have been leveraged to create controllable diffusional sources of soluble chemicals[29]. More recently, cellular responses of cells to gradients in 3D have been the center of attention as they model cancer invasion processes and other diseases more closely. The complexity in the readout induced by migration of cells in 3D[30, 31] can be mitigated by constricting the cells in thin channels producing 2D-like migration in a 3D environment.

1D gradients - The efforts to further simplify the migration environment has resulted in the development of 1D migration platforms[32]. 1D migration channels are defined by a narrow channel in which a single cell can migrate at a time and in some cases the cell fully contacts all 4 surfaces of the channel (Figure 1D). The small scale of these platforms yields the added advantage of generating a convection-free environment. The simplicity of 1D migration systems enable testing of specific hypotheses on gradient sensing by providing multiple paths for a cell with tailored natures and slopes of the gradient[33]. Strikingly, it has been shown that cells migrating in 1D display more similarities in internal cell organization with 3D migrating cells than cells migrating on a 2D surface[34]. The physical constriction of cells appears to be a key aspect of cellular migration[35]. One possible limitation of these gradients is the fact that the shape of the gradient is significantly impacted by the presence of the cell that constricts most, if not all, of the channel. Another limitation is the inability of measuring the directionality of the migration, instead proxy measures are used, such as the percent cells moving in towards the gradient source.

Immobilized gradients, density/stiffness/alignment gradients, electrical gradients - Beyond gradients of soluble factors, a plethora of other types of gradients have been shown to have physiological importance and play key roles in disease pathogenesis. Immobilized gradients are fixed gradients of molecules tethered to a matrix and have been shown to exist, and be one of the modes of action, of chemokines such as CXCR3 ligands[36]. Immobilized gradients have attracted some microscale engineering efforts, noticeably from the perspective of surface chemistry functionalization[37]. These, however remain under-utilized for biological applications, perhaps because of difficulty of creating these types gradients in a 3D migration environment and in a controllable and user-friendly way. Directed cell migration in gradients of matrix density, stiffness, or alignment represent another physiologically relevant type of migration, commonly called haptotaxis. Extracellular matrix density and alignment have been shown to be important predictors of tumor invasion and cancer progression. Stiffness gradients have been shown to induce migration in the absence of other signaling factors and these gradients have been modeled in microscale platforms[38, 39]. Despite the central aspect of biomechanical properties in cancer invasion and cancer cell migration, haptotaxis gradients are under-represented in biological research, potentially due to complex fabrication procedures that are difficult to adopt in biology labs. Finally, migration mechanisms due to electric fields have been documented (galvanotaxis or electrotaxis). These have been shown to have potential applications in regulating immune response to wounds[40] and could be used to improve healing[41].

Biological models

Overarching questions driving the field include understanding mechanisms of gradient sensing and directed cell migration as and their role in human diseases[42]. It is clear that there are multiple mechanisms at play allowing the cell to migrate in different environments and adopt different behaviors in response to specific stimuli[43]. The range of biological models of gradient sensing developed to answer these questions have been closely tied with technological advances. The ability to generate gradients in reliable and precise ways has supported the development of more physiologically relevant models for directed cell migration. These biological models are overviewed here.

Dictyostelium models - The initial models for directed cell migration developed were based on the amoeba *Dictyostelium discoideum* due to its accessibly and ease of use[44-46]. In particular, the gradient sensing machinery of this model organism could be modified using a genetic approach, and this microorganism was able to migrate in the shallow gradients achieved in early platforms. Important achievements were made using *Dictyostelium* models, such as the identification of amplification mechanisms of gradient sensing[47]. The use of *Dictyostelium* has tapered with the increased use of microscale chemotaxis platforms and human cell migration models (e.g. neutrophils and neutrophil-like cells). Their simplicity and our large body of knowledge on these cells, however, allows the production of physiologically relevant computational models to predict modes of cell migrations [48].

Human neutrophils, HL-60s, and PLBs - Human primary neutrophils are the first responders to wounding and microbial infections. As such they need to rapidly respond to cues originating from the microbes themselves as well as cells in the tissue relaying the information. Neutrophils have been and remain a useful model for gradient sensing primarily due to their availability, robustness and velocity of their migration, and their role in human diseases. Neutrophils are a central and intricate part of the immune response, and defects in their ability to perform surveillance results in many autoimmune diseases[49]. Use of neutrophils to answer mechanistic questions is limited as genetic manipulation is impossible (mainly due to the lifespan and general sensitivity of neutrophils) and most secondary immunostaining is complex. The development of human cell lines, such as HL-60s and PLBs, that allow stable transfections and can be differentiated into a neutrophil-like cells with gradient sensing and migratory properties, has resulted in development of methods to identify the role, regulation, and spatial organization of essential proteins involved in human cell migration[50]. However, these cell lines display significant differences compared to human primary neutrophils (e.g. they lack the IL-8 receptor for instance), limiting their use in modeling/studying some diseases. Neutrophil migration is a much more complex than what was thought previously and microfluidic gradient generation platforms have been enabling in highlighting novel behavior of neutrophils. One important finding is the essential ability for wound resolution for neutrophils to migrate away from the location of inflammation, an effect known as retrotaxis[51, 52]. Microfluidic models are also particularly well suited at assessing functional responses of cells challenged with specific stimuli, in the case of neutrophils for instance, the previous challenge by an endothelial cell alters migration patterns[53]. More recently, the identification of polymorphisms in genes related to neutrophil gradient sensing and migration as well as the ability to rapidly sequence genomes of patients and normal/healthy volunteers enables an exciting approach to determining the functional role of genes in human primary cells[54]. Microscale platforms should in theory allow both the identification of the polymorphism of interest and the measurement of functional outputs (such as migration) using a small number of cells. Use of sample-efficient platforms will enable quicker correlations between polymorphisms and heterogeneity in genes of interest and their function.

Cancer cell invasion and collective cell migration - Cancer cell invasion is a complex process that involves multiple cellular mechanisms including gradient sensing and directed cell migration. Cancer cell invasion is elusive/challenging to

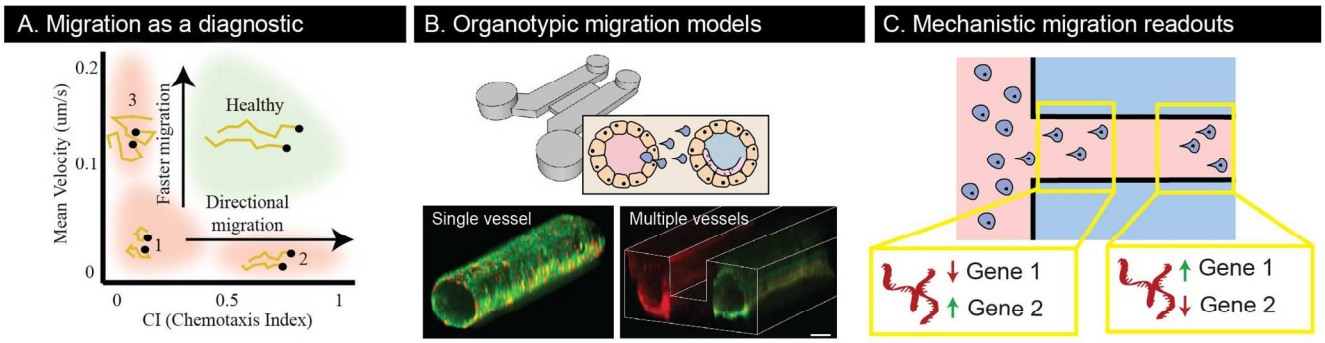

Figure 2: A. Example of utilizing migration readouts for diagnostic purposes. Defects or diseases can cause neutrophils to display an altered migration velocity or directionality. B. Organotypic migration models utilizing lumens lined with epithelial or endothelial cells to model extravasation from the blood vessel and recruitment to a lumen. C. Example of utilizing cell migration readouts to extract and discriminate cells based on migration properties to uncover gene expression and mechanistic information.

understand because of the multitude of interactions occurring in vivo (interactions with multiple cell types, the ECM, and soluble factors) as well as the heterogeneity of the cell populations[55, 56]. It has been shown that gradients of cytokines, growth factors, oxygen, and ECM stiffness/densities can induce directed migration of cancer cells in vitro[57]. The ability of microscale platforms to control spatio-temporal cues is essential for these studies and sets the stage higher complexity models. Cell-sourced gradients, for instance, provide valuable models that can indicate modes of cell migration in vivo[58]. In particular, the ability to alter, degrade, and re-organize the ECM fibers is an integral part of the migration process of fibroblast and epithelial cells. Thus the development of 3D and other microengineered gradients set the stage for physiologically relevant in vitro models of cancer cell invasion[7]. Despite these advances, there is a dearth of platforms allowing the study of migration in controlled matrix alignments. Additionally, the heterogeneity in cell population occurring in human primary and patient samples is an issue of central importance as rare cell populations (e.g. cancer stem cells) can have a transformative effect on the rest of the population [59]. In this context there is a need for platforms that allow the tracking and identification of cancer cell invasion at a single cell level in order to identify the role and characteristics of sub-populations.

Developmental biology and neural guidance. Gradients are a central aspect to developmental biology as their spatial information is paramount for controlling the registration of cells and their differentiation[60]. The nature of the biological model in these studies often requires long term gradients that can last more than several days in stable microenvironmental conditions. For these applications syringe pumps are not practical as they would not allow the slow flows required, necessitate refilling too often, and be unpractical to use in traditional incubators. Systems using osmotic pumping have proven to be advantageous in this context and yield slow, precise, and long lasting flows allowing the study of neural stem cell differentiation[61]. Gradient devices can also be used at a cellular level to direct the growth and orientation of a cell, which is the case of neural guidance for example. Multiple approaches have been investigated so far including soluble factor gradients[62] and immobilized surface gradients[63].

Future directions for gradient sensing platforms

Gradient generation platforms have enabled advances in studies of directed cell migration and several microscale platforms have already became established and commercially available (e.g. the Ibidi and BellBrook Labs platforms). Following these significant advances a number of new research directions have emerged. Here, we provide our perspective on important trends that may transform the field of gradient sensing and directed cell migration.

Migration as a diagnostic - A recent push of gradient generation platforms has been to explore the use of directed cell migration and the associated functional readouts as a diagnostic tool (Figure 2A). At first glance, it may appear that cell migration is an integrated readout that can mask precise mechanistic defects and often only inform at a holistic level. However, it has been shown that defects in neutrophil migration can be an early diagnostic indicating the survival of burn victims [64] and could be a sensitive and quantifiable predictor of asthma[65]. Neutrophil migration platforms have also been demonstrated for the diagnosis of rare genetic mutations that result in leukocyte adhesion deficiencies[23]. Using functional readouts for diagnostics remains complex both in the operation of the microengineered device and the establishment of proper controls currently limiting these to laboratory settings. However, recent microengineering advances in platforms for functional migration readouts have established methods to interface with the device that does not require complex equipment and can be performed with a single drop of blood[29, 66]. Further, platforms such as the Kit-On-A-Lid Assay platform aim to create entirely pre-packaged kits that do not require fluidic handling equipment[67]. Gradient sensing and directed cell migration platforms also show promise for personalized medicine approaches. Specifically, a dream of the cell migration community is to use these platforms to screen for

pharmaceuticals that disrupt cancer invasion processes on a per patient basis. The use of microfluidics will enable the screening of a wide range of drugs while requiring limited patient sample[68].

Organotypic models – The migratory characteristics of cells are strongly affected by the structure of the microenvironment. As such, the models developed thus far have been informative but do not accurately capture the complex architectures in which cells migrate and receive signals in vivo. The rise in organ-on-a-chip and, more generally, organotypic microscale models, have set the stage for a whole panels of clinical discovery opportunities. In the context of the lung for instance, current animal models have been put in question and in some cases appear limited and non-predictive[69]. The ability to use human cells in a relevant environment has the potential to replace or complement animal models. To dated, several organotypic models have been developed that have high potential to better mimic cell migration environments. Lung-on-a-chip and skin models, in particular, have highlighted the potential of microscale models[70]. The recruitment of immune cells to the lung environment or to a wound would be useful models that have yet to be developed. The generation of lumens and in vitro blood capillaries are another area of interest which will yield important insights into cell migration, particularly for oncology applications (Figure 2B, e.g. liver cancer[71], breast cancer[72], bonne marrow lymphoma[73]). Lumen-based organotypic models have the ability to reproduce interfaces between a 2D environment and a 3D environment in a situation where form imparts function (i.e. the shape and size of the lumen is important)[74]. Additionally methods for lumen formation are being developed that are compatible for use in biology labs and for screening applications[74-78]. Among the many potential applications, these models will allow the study of cell-sourced gradients, which reproduce the complex cocktail of cytokines, growth factors, and small molecules that induce and inhibit cell migration in various disease states including wounds, auto-inflammatory diseases and cancer. In cancer, the ability to replicate the metastatic site along the vasculature can lead to new mechanistic insights about the regulators of invasion[79].

Cell migration is not the only useful readout - Emphasis has been placed on measuring characteristics of the directed cell migration as the endpoint of most of the current migration platforms developed. Typical readouts include the chemotactic index, migration velocity, and cell morphology. Because migration is an integrated phenomenon, these readouts inform little on the underlying mechanistic phenomena causing the migration defect. It is clear that obtaining mechanistic information at the nucleic acid or protein level will yield important information for fundamental science and diagnostics[80]. Different potential paths forward can be imagined. A simple approach correlating information obtained by two platforms, such as an omics platform and a gradient generation / migration platform might be used to deduce information on how gene expression and gene function correlates with the migration readout. A more exciting approach is using the directed cell migration platform as a front end for selecting or clustering specific cells of interest (Figure 2C). These cells would then be extracted from the platform and mechanistic information could be determined through gain and loss of function experiments and additional biochemical readouts. In the latter example, the gradient sensing and directed cell migration are not the final readout, rather a means to induce a desired reaction from a cell of interest in order to subdivide a population of cells and measure a defined response (e.g. gene or protein expression).

Conclusions

Significant progress has been made over the last decade in the area of microengineered platforms for generating gradients and studying directed cell migration. In fact the intrinsic ability to use diffusion to generate gradients made microfluidics an immediate and enabling technology for these applications. We are beginning to see the transition between punctual collaborations between biology and engineering labs to more established commercialized platforms. Now that the foundational physics and engineering concepts have been demonstrated, a second wave of microscale migration platforms are emerging. Two general trends that these platforms display are (1) an increased clinical and physiological relevance though the rise of organotypic models and (2) the search for translational medical applications with the development of diagnostic tools based on functional readouts of cell migration. Down the line, efforts are needed towards engineering simple organotypic models to fuel this second wave of migration platforms as well as tools to obtain more mechanistic information about the migrating cells.

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