Studying Guidance Cues in Collective Cell Migration: Tools and Metrics

by

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Directed cell migration is essential in morphogenesis, regeneration and pathological conditions. Of particular interest is collective cell migration, in which groups of cells migrate in a coherent and coordinated manner while remaining in contact with one another. In vivo, migratory cells are exposed to a complex signalling milieu that directs their migration; however, how cells prioritize and translate these cues into directed migration is not fully understood. In vitro systems simplify the systematic study of the effect of guidance cues in cell migration by providing increased flexibility to manipulate these cues. This work describes novel in vitro tools and analytical metrics for the analysis of collective cell migration and utilizes them to probe the effects of combinations of guidance cues on cell migration. This includes an improved analytical methodology for the classical scratch wound assay in which individual cell migratory behaviour is characterised with high spatial and temporal resolution; a novel metric designed specifically for measuring cell-cell coordination during collective cell migration is also suggested. The tools described in this work were used to investigate the mechanism for propagation of guidance cues in epithelial cells. Migration guidance from topographical features was found to propagate to cells not directly exposed to them through mechanical interactions between cells not mediated by cell-cell junctions or intracellular tension, but arising rather from volume exclusion.
Additionally, the tools proposed were also used to characterise the effect of modifying the expression of the Planar Cell Polarity signalling protein Vangl1 on the collective migration of endothelial cells. Vangl1 expression was found to influence the migration of endothelial cells by modulating directedness in the scratch wound assay, but not in a variety of other migration assays, implying damage signals from the scratch were necessary for a Vangl1 migration phenotype. The work presented here is a first step to an improved experimental and analytical pipeline to understand the effect of combinations of guidance cues on directed collective cell migration.
To my parents, Carolina Ferroni and Rodrigo Londoño, whose sacrifices paved the way here.
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# List of Abbreviations

- $\alpha$: migration angle with respect to migration direction in the previous timeframe
- 2D/3D: two-dimensional/three-dimensional
- AFM: atomic force microscopy
- ankrd6: Diversin (mammalian Diego homolog)
- ANOVA: analysis of variance
- aPKC: atypical protein kinase C
- AVE: anterior visceral endoderm
- BB: blebbistatin
- Ca2+: Calcium
- CalA: calyculin A
- Cdc42: cell division control protein 42
- cDNA: complementary deoxyribonucleic acid
- Celsr: cadherin EGF LAG seven-pass G-type receptor (mammalian Flamingo homolog)
- CFDA: carboxyfluorescein diacetate succinimidyl ester
- CHO: Chinese hamster ovary
- CIHR: Canadian Institutes of Health Research
- CIL: contact inhibition of locomotion
- cm: centimeter
- CO2: carbon dioxide
- cos: cosine
- Ctrl: control
- CXCL12: C-X-C motif chemokine 12 (also known as SDF1)
- CXCR4: C-X-C chemokine receptor type 4
- Daam1: Dishevelled-associated activator of morphogenesis 1
- DAPI: 4',6-diamidino-2-phenylinodole
- Dg: Diego
- DIC: differential interference contrast
- DSL4: delta-like ligand 4
- DMEM: Dulbecco's Modified Eagle Medium
- DMEM/F12: DMEM with nutrient mixture F12
- Dsh/Dvl: Dishevelled
- EBM: Endothelial Basal Media
- EC: endothelial cells
- E-Cad/E-cadherin: Epithelial cadherin
- ECM: extracellular matrix
- EDTA: ethylene diamine tetraacetic acid
- EGM: EGM-2 supplemented with EGM-2 Bullet-Kit
- EGTA: ethylene glycol tetraacetic acid
EMT epithelial-to-mesenchymal transition
ERK extracellular signal-regulated kinases
E-SFM endothelial serum-free media
ExE extraembryonic ectoderm
FACS flow activated cell sorting
FBS fetal bovine serum
FGF/bFGF fibroblast growth factor/basic FGF
FITC fluorescein isothiocyanate
Fmi Flamingo
Fz Frizzled
GAPDH glyceraldehyde 3-phosphate dehydrogenase
GFP green fluorescent protein
h hour
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP horseradish peroxidase
HUVEC human umbilical vein endothelial cells
JAK/STAT Janus kinase/signal transducers and activators of transcription
µg microgram
µL microliter
µm micrometer
MAPK mitogen-activated protein kinases
MDCK Madin-Darby canine kidney
mL milliliter
mM millimolar
mRNA messenger RNA
MSD mean squared displacement
MTOC microtubule organizing centre
MTS Monte-Carlo time steps
mW milliwatts
NC neural crest
N-Cad/N-cadherin Neural cadherin
ng nanogram
NSERC National Science and Engineering Research Council
OGS Ontario Graduate Scholarship
P0 Passage 0
Par partitioning defective protein family
PBS phosphate buffered saline
PCP planar cell polarity
PCR polymerase chain reaction
PDGF platelet-derived growth factor
PDMS polydimethylsiloxane
PECAM platelet endothelial cell adhesion molecule
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Pen/Strep</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>Pk</td>
<td>Prickle</td>
</tr>
<tr>
<td>PNAS</td>
<td>Proceedings of the National Academy of Sciences</td>
</tr>
<tr>
<td>PPIA</td>
<td>peptidylpropyl isomerase A</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>Rac</td>
<td>Ras-related C3 botulinum toxic substrate</td>
</tr>
<tr>
<td>Rho</td>
<td>Ras homolog gene family</td>
</tr>
<tr>
<td>RIE</td>
<td>reactive ion etching</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigmented epithelium</td>
</tr>
<tr>
<td>RPLP0</td>
<td>ribosomal protein, large, P0</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>s second</td>
<td></td>
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<tr>
<td>SDF1</td>
<td>stromal cell-derived factor 1 (also known as CXCL12)</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean or scanning electron microscopy depending on context</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>sin</td>
<td>sine</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>Si-wafer</td>
<td>silicon wafer</td>
</tr>
<tr>
<td>Θ</td>
<td>migration angle with respect to a vector orthogonal to the wound</td>
</tr>
<tr>
<td>TCPS</td>
<td>tissue-culture polystyrene</td>
</tr>
<tr>
<td>TNFC</td>
<td>Toronto Nanofabrication Centre</td>
</tr>
<tr>
<td>TPMR</td>
<td>Training Program in Regenerative Medicine</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethyl rhodamine iso-thiocyanate</td>
</tr>
<tr>
<td>Vang/Vangl</td>
<td>Van Gogh/Van Gogh-like</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF/VEGFR</td>
<td>vascular endothelial growth factor/VEGF receptor</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula Occludens-1</td>
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Chapter 1
Introduction and Motivation

1.1 Motivation

Cell migration is an absolutely essential process for the developmental and adult phases of an organism. The patterning of the body plan depends on the properly directed movements of large numbers of cells\(^1\); errors in migration often result in severe birth defects at best or embryonic lethalities at worst\(^2\)\(^-\)\(^4\). Even after birth, cell migration continues to play key roles in growth, regeneration and homeostasis, and is also an important process for cancer invasion and growth\(^5\). Because of how critical cell migration is to an organism’s life, especially directed cell migration during early development, it is important to understand the mechanisms that guide the migratory behaviour of cells.

The migration of single cells has been under study for more than a century, and we understand a number of the variables that affect it such as cell-substrate adhesion, the cellular environment (including extracellular signals), and the localization and activation of multiple proteins\(^6\)\(^,\)\(^7\). However, until recently, studies of the migration of large cell collectives (in which the cells must cooperate with their neighbours) were prohibitive from both data acquisition and data analysis standpoints. Recent technological advances have made it possible for scientists to study collective cell migration and have allowed us to understand that the behaviour of cells migrating collectively is not simply an extension of the behaviour of single cells, but rather, that cell-cell interactions create emergent behaviours that could not have been predicted from single cell data\(^8\)\(^,\)\(^9\). That migratory cells affect the migration of their neighbours (and hence the discovery that migrating collectives possess inherent guidance mechanisms) has made the question of how cells prioritize or integrate different guidance cues more pressing than ever before.
Figure 1.1 – Guidance cues during cell migration: As cells migrate, they are exposed to a variety of signals that affect their migratory behaviour. These signals include those from other cells, which may be chemical or mechanical in nature; signals from the environment, such as topographic features, substrate stiffness, adsorbed molecules, external forces, or free space; and other external cues such as chemical or electrical gradients. Each cell must prioritize/integrate these signals in order to migrate.

In vivo, cells are exposed to multiple guidance cues at the same time (Figure 1.1) and their proper behaviour often depends on their responses not to any particular signal, but rather to their combination. Research in corneal wound healing, for example, has shown that electrical fields and substrate topography combine to drive the re-epithelialization process after injury\textsuperscript{10}. Similarly, the migration of neural crest cells has been shown to depend on their ability to process chemotactic signals\textsuperscript{11} in combination with signals arising from mechanical interactions with other cells\textsuperscript{12}. In both of these systems, as well as in many others, the lack of any particular cue results in abnormal phenotypes, which indicates the final outcome is dependent on the cells’ responses to all of these signals in combination.

While our imaging and computational resources have advanced rapidly, the assays and metrics we use to describe cell migration have been left behind and seen only limited innovation. Because of how complex cell migration is, most studies focus on a single variable at a time to avoid confounding the results. The information gathered in this manner is both useful and important, but in the same way that the dynamics of single cells migration are now known not to be predictive of the behaviour of cells in collective, it is likely that synergistic effects between guidance mechanisms have larger impacts than we can currently predict. This highlights the
importance of both acknowledging and manipulating cell migration guidance mechanisms in combination.

Additionally, the metrics currently in use fail to fully take advantage of the migration data that we are able to gather. Some of the more common descriptors are metrics that were developed to describe the behaviour of inert particles\textsuperscript{13,14}. The similarities between multi-particle systems and cell collectives make it so that these metrics are not entirely unsuitable for cell migration studies, but the differences between the systems mean that existing metrics are unable to capture cell-specific behaviour\textsuperscript{15,16}. For example, metrics such as correlation length are appropriate in systems with rotational symmetry, but cell migration is a polarized behavior, which impacts the behaviour of other cells along the direction of migration much differently than it does that of cells located perpendicularly to the migrating cell\textsuperscript{17}.

The work contained in this thesis seeks to illustrate the importance of developing new \textit{in vitro} assays and metrics to improve our understanding of collective cell migration and the guidance mechanisms that underpin it \textit{in vivo}. Further, this thesis aims to explore the effect of multiple guidance cues on cell migration, particularly in regards to non-intuitive outcomes.

1.2 Overall Thesis Goal

The motivation for the work presented here was to develop and use novel tools to visualize and discern the effect of combinations of guidance cues on collective cell migration. Additionally, individual hypotheses form the basis for the work contained in some of the chapters:

\textbf{Chapter 4}: Mechanical cell-cell interactions are sufficient for propagation of guidance signals.

\textbf{Chapter 6}: PCP signalling depends on wound-related signals to affect endothelial cell migration.

1.3 Thesis Objectives

The main objectives of this thesis were:

1. To develop new tools to improve the study of collective cell migration. This included both the development of new metrics and the creation of enhanced analysis pipelines for existing migration assays. In particular, we aimed to:
• Increase our understanding of collective guidance mechanisms by developing new metrics that measure the effects of cell-cell interactions (Chapter 3 and Chapter 4)

• Improve the analysis of the classical wound healing assay by using cell tracking techniques to understand spatial and temporal heterogeneities (Chapter 5)

2. To use these tools to study the impact combinations of specific guidance mechanisms have on cell migration. To do so, we chose to use our tools to study in detail how:

• The combination of two guidance mechanisms (topographic cues and cell-cell interactions) results in emergent behaviours, including the propagation of topography-induced biased to cells not directly exposed to topographic signals (Chapter 4).

• Planar Cell Polarity signalling affects cell migration within specific contexts, particularly looking at the effect of cell-cell interactions and mechanical guidance mechanisms, and how individual cell behaviour is affected differently by PCP signalling depending on context (Chapter 6).

1.4 Thesis Overview

This thesis contains 7 chapters. In Chapter 2, I provide a review of what is presently known about cell migration and its guidance mechanisms, as well as common cell types, assays and metrics currently in use for the study of collective migration. Chapter 3 describes a novel algorithm for measuring cell-cell coordination during collective cell migration. Chapter 4 demonstrates that combining guidance mechanisms results in augmenting the impact of both and that cell-cell interactions can propagate guidance cues to cells not directly exposed to them. In Chapter 5, I describe an enhanced analysis method for the classical scratch wound healing assay and provide evidence that this new method can increase our understanding of collective cell migration phenotypes. Chapter 6 examines, in a more relevant system of primary endothelial cells, how different guidance mechanisms integrate with each other, and how the effect of any specific cue may depend on others, using the methodology described in the previous chapter. Lastly, Chapter 7 provides final conclusions on the work contained in this thesis, highlights the significance of the contributions to the field and introduces questions for future work in this field.
1.5 References


Chapter 2
Literature Review

2.1 Introduction

Cell migration is an important, but poorly understood biological process. Cell migration is necessary for immune responses, wound healing and plays a role in cancer progression. It is central to morphogenesis and the developmental process, but also to the biology of adult organisms. However, much about how cell migration is accomplished and coordinated is still not understood.

![Figure 2.1 – Modes of collective cell migration:](image)

Cells may migrate collectively in A) slugs, as in the lateral line primordium in zebrafish, B) 2D sheets, as they do in wound healing, C) in small groups, like border cells during Drosophila oogenesis, or D) they may extend branches or sprouts from existing structures. Modified with permission from 1.

Cells can migrate either separately as single cells, or cohesively in groups. The term “collective cell migration” is often used to refer to the latter, and encompasses a variety of types of “collective migration.” In collective cell migration, each cell is known to affect the migration of its neighbours. In order for migration to occur, the movement of all the cells must be coordinated. In the context of this thesis, collective cell migration will be used to refer exclusively to situations where cells maintain their connections through cell-cell junctions as they migrate (Fig. 1). For example, epithelial cells are known to remain tightly connected as they move. Depending on the situation, they might move as strands, chords or “slugs” (Figure 2.1A), sheets (Figure 2.1B), as small cell groups (Figure 2.1C), or extend branches or sprouts from existing structures (Figure 2.1D)2. Others use collective cell migration to refer to any situation in which cells migrate in the same direction1. One such case is that of neural crest (NC) cells, which migrate as a very loosely associated group, with individual cells moving mostly independently but affected by contact with other cells3.
2.2 How Cell Migration is Accomplished – The Cellular Scale

Figure 2.2 – Cell migration is a cyclical process: Cell migration requires that cells first extend a protrusion (lamellipodia), which becomes anchored to the substrate after the formation of a new focal adhesion. This allows the cell to contract, moving the cell body forward. The cell then retracts its rear to complete one cycle. Repeating these actions results in cell migration.

Individual cell migration occurs through one of two modes: amoeboid migration, which is driven by cell expansion and contraction, is characterised by weak cell-substrate interactions, and results in rapid movement\(^4\); and so-called mesenchymal migration, which is slower and depends on strong cell-substrate adhesions. The process of protrusion extension and generation of traction forces differs strongly between these two migratory strategies, and though several cell types are able to switch between them\(^5\), this thesis focuses on the mesenchymal mode exclusively.

The process of mesenchymal single cell migration has been separated into 5 distinct steps (Fig 2.2): First, a cell breaks symmetry and polarizes, establishing a front and a rear. Second, the cells extends protrusions (lamellipodia and filopodia) forward from the front (in the direction of motion). Third, the protrusions adhere to the substrate, forming an anchoring point. Fourth, the cell body contracts and translocates forward. Lastly, attachments at the rear of the cells are
released and the tail-end of the cell retracts. As these actions repeat in the same general
direction, the cell achieves directional migration. This is a consequence of directional sensing,
but is not necessary for cells to migrate randomly. In the absence of cell polarization, or when
there are issues with protrusion extension or stability, cell-substrate adhesion, or the ability of the
cell to dissolve its rear adhesions and retract the trailing end, a cell is unable to migrate.

A non-motile, non-polarized cell is radially symmetric in its morphology and cytoskeletal
distribution. Cells appear to first lose radial symmetry by changes in cytoskeletal organization
that define a cell rear, which are followed by morphological definition of the rear and rear
retraction and then by extension of protrusions at the front, though this may depend on cell
type (and potentially on the presence of directional cues). These initial differences are then
reinforced by feedback loops that act to restrict “front” behaviour to a single site on the cell
body. Regardless of what the original symmetry-breaking step is, the establishment of front-
rear polarity has been shown to be essential for cell migration. In many cell types, the nucleus
moves rearward. When this does not occur, these cells are unable to migrate.

The extension of protrusions from the cell body is also essential. Part of the polarization process
involves limiting protrusion extension to only the front of the cell. Logically, it is easy to
imagine how cell migration would require protrusion extension, and only in one direction;
however, protrusions that grow too rapidly or do not stop growing inhibit migration as well.
One of the issues with the extension of too many protrusions, or with protrusions that extend too
rapidly and fail to stop growing is that it prevents the maturation of these protrusions. This is due
to the fact that the nascent focal complexes that form the adhesions in growing protrusions
require mechanical forces to coalesce into larger, mature adhesions.

Mature focal adhesions are necessary for the next step of cell migration, as they provide enough
resistance to support cell body translocation and prevent membrane contraction. The forces
involved in cell contraction cannot be borne by weak cell-substrate adhesion, which prevents cell
traction and hence movement. Cells unable to retract their rear are sometimes able to migrate,
leading to a long tail. However, migration is hampered. When cells migrate collectively, each
cell must undergo the same steps as the single cells above. However, they must also coordinate
their motion and behaviour to maintain tissue cohesiveness.
2.3 How Cell Migration is Accomplished – The Molecular Scale

A number of proteins are involved in cell migration. The process is complex, and not fully understood. Some of the most important players in migration are cytoskeletal proteins, molecular motors (particularly myosin II), the Rho family of GTPases, integrins and other focal adhesion complex molecules, and in the case of collective cell migration, cell-cell adhesion molecules including cadherins.

2.3.1 The Cytoskeleton in Cell Migration

Actin is potentially the most important molecule in cell migration. The polymerisation and depolymerisation of globular actin (G-actin) into actin filaments (F-actin) is thought to drive the extension of lamellipodia and filopodia. Additionally, actin associates tightly with the molecular motor myosin17. This actomyosin network is responsible for generating the contractile forces that move the cell body forward after protrusions have attached18.

Researchers have found that cell motility is driven by the actions of two separate actin networks: a highly dynamic lamellipodial one, located in the 1-3μm closest to the edge of the membrane, characterised by rapid retrograde flow (a well-known phenomenon where individual actin fibers constantly move towards the back of the cell19) and actin assembly and disassembly, which appears to have an exploratory function, and results in the extension of transient, random, short protrusions; and a more stable lamellar one, which extends through the rest of the cell protrusion, is characterised by slower retrograde actin flow and a punctate pattern of actin assembly and disassembly, and is responsible for persistent and productive movement of the cell membrane20. Despite this distinction between lamella and lamellipodia, in this work, like many others, lamellipodia is used to refer to the full protrusion which consists of both.

Recently, new evidence has come to light that epithelial cell polarization might be driven by competition between these two networks and the even more stable cortical actin network. The sequestration of actin monomers in the cortical actin network, which has much lower turnover, limits the amount of G-actin available for polymerisation of F-actin in protrusions, and hence prevents lamellipodial extension. This inhibits cell polarization because extension of protrusions increases retrograde actomyosin flow, which in turns increases the relative quantity of myosin that is located at the rear of the cell and leads to contraction, establishing the cell rear and leading
to stable cell polarization\textsuperscript{21}. Further, actin helps define cell polarization in other ways. For example, retrograde actin flow also helps move the nucleus rearward, which defines the direction of cell motion\textsuperscript{22}.

Actin fibers are also critical for the transmission of the forces necessary for cells to move, and in collective cell migration, the actin cytoskeleton is believed to participate in the coordination of behaviour across cell boundaries\textsuperscript{16}.

The involvement of intermediate filaments in cell migration is not yet well understood. However, mesenchymal cells, as well as cells undergoing epithelial-to-mesenchymal transitions (EMT)\textsuperscript{23} show increased expression of vimentin, one of the proteins that makes up intermediate filaments. Further, downregulating vimentin in cancer cells has been shown to inhibit their migration\textsuperscript{24,25}.

Microtubules are also important players in cell migration; they are particularly involved in the process of cell polarization\textsuperscript{26,27}, and in the establishment of persistent directed migration. Two pools of microtubules (the stable and dynamic pools\textsuperscript{28}) are involved in different aspects of cell migration. Stable microtubules are known to be involved in the turnover of focal adhesions\textsuperscript{29}; they appear to “mark” focal adhesions for dissolution\textsuperscript{30}, and are necessary for the retraction of the tail in migrating cells\textsuperscript{31}. The dynamic microtubule pool, on the other hand, is responsible for cell polarization\textsuperscript{32,33}. Dynamic microtubules maintain the position of the microtubule organizing center (MTOC) when the nucleus moves rearward during polarization. This is necessary for directional sensing and directed migration. Without this pool of microtubules, the cells are unable to close wounds or respond to chemical cues. They are also responsible for the distribution of E-cadherin to cell-cell junctions at the cell membrane.

The interaction of microtubules and actin is also important for cell migration. Actin retrograde flow causes destabilizes microtubules\textsuperscript{34}. The polymerisation and depolymerisation of microtubules in turn regulate actin assembly and disassembly at the leading edge\textsuperscript{35}.
2.3.2 The Rho GTPases in Cell Migration

GTPases are molecular switches which, when turned on, effect change in target proteins. The Rho family of GTPases controls a variety of processes in cells ranging from vesicular transport to cell cycle progression and from gene expression to cell shape. Though several members of the family have been implicated in cell migration, the most important players are Rac, Rho and Cdc42 (Fig. 3).

Rac, particularly Rac1, is essential for cell migration: it affects the extension of protrusions by controlling the polymerisation of actin filaments. This is done through a number of different mechanisms. Rac is responsible for the formation of a branched actin network as it, through Scar, activates the actin-nucleating protein complex Arp2/3, which creates new branches at an angle to existing ones. It is also able to uncap the capped ends of existing actin filaments, which creates a free barbed end and results in extension. Rac may also promote depolymerisation of actin, leading to the release of G-actin monomers and making them available for polymerisation.

There is some evidence that antagonistic interactions between Rac and Rho can also affect cell migration by localizing cell contractility and cell-substrate adhesion.

The role of Rho is slightly less well-defined. It controls cell contractility, which is important for both the formation of focal adhesions, and hence the strength of cell-substrate interactions, and the formation of stress fibers and cell body translocation. Rho may also affect focal adhesions through the stabilization of microtubules. Further, Rho also inhibits the
depolymerisation of actin by cofilin, leading to the formation of stress fibers14,44. Rho has additionally been implicated in the retraction of the cell rear, though this may be a cell-specific phenomenon45 or may be mediated by other signals in confluent sheet migration39.

Cdc42 is central to cell polarization in cell migration as it controls the nuclear translocation that helps define the front and rear22,46. Cdc42 inhibition leads to the random extension of cell protrusions all around the cell periphery instead of just at the front39. This is consistent with a role for Cdc42 in chemotaxis, as cells need to establish a clear polarity to respond in a directed fashion.

2.3.3 Cell-Substrate and Cell-Cell Interactions and Their Importance to Cell Migration

Cells attach to the substrate using integrins, which are heterodimers composed of an α and a β unit47. Depending on the combinations of these units, integrins have more or less affinity for particular extracellular matrix (ECM) molecules; for example, the migration of endothelial cells (EC) on collagen is mediated by α2β1, whereas on vitronectin αvβ3 dominates48. Integrins have an important role in stabilizing nascent protrusions by anchoring them to the substrate49. These early adhesions are termed focal complexes, and their formation is regulated by Rac and Cdc4250. These complexes are either turned over or mature, in a Rho-dependent process, into larger, more complex structures termed focal adhesions51, which are responsible not only for adhesion to the substrate but also for the transduction of various signals50. The speed of cell migration has been shown to depend on both the concentration of extracellular matrix molecules and on amount of expression of the particular integrin molecules52, with a biphasic relationship that shows low cell speed at both low and high ECM concentrations, and maximal speed at an intermediate combination of both ECM and integrin expression48. However, this dependency is further modulated by actomyosin contraction, which indicates that feedback between these two mechanisms may be more important to cell migration45,50.

In collective cell migration, the cells’ interactions with their substrate continue to be important. However, each cell’s interaction with other cells has an equally large impact on migration through both mechanical interactions and cell-cell signalling53. Adherens junctions, which are cadherin-based, are heavily involved in cell-cell signalling, and play a big role in directing collective cell migration in a number of ways. The tugging on cadherins by other cells can
reorient the direction of protrusion extension, altering the direction of migration in leader cells\textsuperscript{54}, inhibit the formation of protrusions in follower cells\textsuperscript{55}, and potentially coordinate globally polarized Rac activity\textsuperscript{56}, resulting in improved directional sensing for the whole collective. In mesendodermal cells, cells lacking cadherins which migrate as part of a group lose directionality compared to single cells; cells with normal cadherin levels migrate in a similarly directed fashion as single cells do\textsuperscript{57}. Cadherin junctions have also been shown to greatly increase the directed migration of cells exposed to electric fields\textsuperscript{58}, such that cells within confluent sheets migrate in a much more directed manner than cells within small clusters or single cells. In fact, the directedness of motion appears to correlate with the number of cells migrating in a group\textsuperscript{58}, which reinforces their importance in collective cell migration. Interestingly, however, increased levels of cell-cell adhesion molecules have been found to interfere with and ultimately halt collective migration\textsuperscript{59}. In a model of mammary morphogenesis in female puberty, which requires the collective migration of mammary duct epithelial cells, the apicobasal polarity of the epithelial cells is lost and cell-cell junctions (particularly tight junctions) are inhibited, but not completely; the areas with some remaining junctions are highly motile\textsuperscript{60}. Moreover, junctions are re-established upon completion of migration, indicating that regulation of cell-cell adhesion is critical for collective migration. Together, this evidence indicates that regulating the strength of cell-cell adhesion is critical for collective cell migration, as both complete abolishment and the lack of down-regulation of cell junctions inhibit collective cell migration.

Another tightly regulated aspect of cell-cell contacts is their actomyosin tension. Various factors have been shown to play a role in decreasing actomyosin tension across cell-cell junctions in migrating collectives including polarity-related proteins\textsuperscript{16} and myosin isoforms\textsuperscript{61}. Failure to do so hinders migration and often results in cell scattering. However, cell-cell contacts still maintain significant tension, and are, in fact, able to support the migration of cell “bridges” over non-adherent areas\textsuperscript{62}. That cell collectives are able to migrate as a group despite having within them non-migrating cells, such as these sheets over non-adherent substrates\textsuperscript{62} or the polar cells within border cell clusters in \textit{Drosophila}\textsuperscript{63} is testament to how critical cell junctions are to collective migration.
2.4 How Cell Migration is Accomplished – Directional Guidance Mechanisms

The formation and growth of the body plan requires that all morphogenetic processes be tightly regulated. Migration is no exception, and multiple mechanisms exist that control where cells migrate to. Depending on context, one guidance mechanism may dominate over others, or several may coordinate to ensure cells reach their intended target.

2.4.1 Contact Inhibition of Locomotion

One of the first cell migration guidance mechanisms discovered was contact inhibition of locomotion (CIL)\textsuperscript{64}, which refers to cells changing their migration direction when coming into contact with each other (or other barriers). This occurs through the collapse of existing protrusions and extension of new ones at different locations in the cell membrane\textsuperscript{65}. Though this is the typical response of non-cancerous cells, it is abrogated in cancer cells when meeting non-cancerous ones, which contributes to their invasive capabilities\textsuperscript{66}. How CIL is implemented in cells is still not fully understood, but certain embryonic patterns are known to depend on CIL, with defects appearing if cells are removed\textsuperscript{67}.

Recent work has shed light on some of the molecular mechanisms that underlie CIL: Both increasing and decreasing Rac1 activity and increasing Rho activity result in increased invasiveness of heterotypic fibroblasts\textsuperscript{68}. However, since the localization of those is just as important as their activity, this is not a surprising result. However, two important signalling modules (the Planar Cell Polarity (PCP) signalling pathway and Eph/Ephrin signalling) that are known to mediate collective behaviours have been implicated in localizing the Rho GTPase activity that leads to protrusion collapse and subsequent extension in a different location.

The Planar Cell Polarity (PCP) signalling pathway consists of six core proteins – Frizzled (Fz), Dishevelled (Dsh), Prickle (Pk), Van Gogh (Vang), Flamingo (Fmi) and Diego (Dg), which affect actin organization and coordinated, polarized multi-cellular behaviours in various systems. The details of how these proteins interact and result in polarized behaviours in multiple different context are not clear; however, PCP signalling has been shown to affect the CIL of neural crest (NC) cells by localizing RhoA activity to cell-cell contacts, which results in protrusion retraction\textsuperscript{69}. The polarity protein Par3 has also been implicated in CIL. In NC cells, Par3
localizes to cell-cell contacts, inhibiting the activity of Rac1, which leads to protrusion collapse by inhibiting microtubule polymerisation\textsuperscript{70}. Others have also shown that not only is microtubule collapse necessary at the contact site, but also that new microtubules are polymerized at the site of the new protrusion\textsuperscript{71}. These studies suggest that polarity-related proteins, as well as microtubules, which are implicated in the establishment of polarity, are important players in CIL, and are likely affected in invasive cancers.

Eph/Ephrin signalling has gained significant prominence in recent years for its potential in cell-cell signalling. One of the most important features of Eph/Ephrin signalling is that it is bi-directional, which means contact between a receptor and ligand can trigger signalling in both cells. Eph/Ephrin signalling plays an important role in boundary formation by mediating repulsive interactions between otherwise similar cells (Javaherian et al., manuscript submitted). Experiments with prostate cancer cells have shown that Ephrin-A signalling causes repulsion (potentially through activation of RhoA as in PCP signalling), but that signalling between EphrinB2 (in fibroblasts) and the EphB3 and EphB4 receptors (in cancer cells) activates Cdc42 and increases migration without causing the protrusions to collapse; the ratio between these two signalling events determines whether protrusions collapse after cell collisions\textsuperscript{72}.

2.4.2 Migration along a Chemical Gradient – Chemotaxis and Haptotaxis

That cell migration can be guided by chemical signals was first discovered in the 19\textsuperscript{th} century in studies of immune cell behaviour\textsuperscript{73}. Since then, many different proteins, particularly growth factors, have been found to act as chemoattractants to cells, and chemotaxis (the migration along soluble chemical gradients) and haptotaxis (migration along gradients of bound chemicals) have been established as critical cell behaviours during embryonic development. Additional control to chemotactic movement seems in many cases to be provided by antagonistic signalling that repels cells.

The movement of cells during gastrulation is under tight control because errors can result in major defects in adult organisms. Transplantation studies in chick embryos have found that the migration of epiblast cells, which leads to the formation of mesodermal and endodermal structures\textsuperscript{74}, is governed by an attractive gradient of fibroblast growth factor (FGF) 4 in combination with a repulsive gradient of FGF8\textsuperscript{75}. FGF4 has also been shown to play an
important role in the migration of mesenchymal cells in the developing limb bud; the addition of an FGF4-secreting bead results in an ulna that is much shorter than controls as exogenous FGF4 competes with the endogenous signal\textsuperscript{76}.

Other critical morphogenetic events are also controlled by chemotaxis, such as the formation of the trachea\textsuperscript{77} or the migration of the border cells\textsuperscript{78}, primordial germ cells\textsuperscript{79,80}, and neural crest cells\textsuperscript{81}. Angiogenesis, for example, is driven by the presence of vascular endothelial growth factor (VEGF), which has a potent chemotactic effect on endothelial cells\textsuperscript{82-85}. There is some evidence that this gradient is more attractive when the VEGF isoform used is one that tends to be bound to either cells or ECM (eg. Those that interact with heparan sulfate like VEGF164 and VEGF188\textsuperscript{86}) as opposed to a soluble one, suggesting haptotaxis and chemotaxis may both be at play in the guidance of angiogenesis.

Cancer invasion is also influenced by both haptotaxis and chemotaxis. In addition to producing angiogenic molecules to drive endothelial cell migration to tumours\textsuperscript{87}, promoting their growth, tumours also promote their own chemotactic invasion. Chemoattractants secreted by tumour-associated macrophages stimulate the migration of cancer cells and vice versa, enhancing the invasion of cancer into surrounding tissues\textsuperscript{88}. Haptotactic cues, on the other hand, have been shown to selectively increase the migration of invasive subpopulations\textsuperscript{89}.

Haptotaxis is also central to development. Guidance cues secreted by an amphibian ectodermal layer onto a substrate can guide the migration of mesodermal cells. While the secreted matrix is fibrillar, which may be expected to restrict migration to occur along the fibres’ axes, the mesodermal cells actually migrate mostly in one direction, indicating that other cues result in directional migration\textsuperscript{90}. This directional migration is explained by the presence of immobilized platelet-derived growth factor (PDGF) on the secreted matrix\textsuperscript{91}.

2.4.3 Topographic Guidance and Durotaxis

Topographic guidance and durotaxis (the migration along a stiffness gradient) both refer to mechanical guidance mechanisms that depend on substrate differences. While they each refer to different changes to the substrate, most current methods to create stiffness gradients also modify the geometry/chemistry that is available for cell attachments, which mediates topographic guidance. However, \textit{in vitro} experiments have shown that cells migrate preferentially toward
stiffer substrates, though this preference is only applicable until the stiffness reaches approximately 10nN/μm, suggesting cells do not sense stiffness differences at higher values.92

Our current understanding of contact guidance suggests that it arises because anisotropic substrates limit the potential sites where a cell might form adhesions, which results in non-random locations for focal adhesions. Since focal adhesions are necessary for the stabilization of protrusions, this anisotropy biases the direction of stable protrusions, which in turn biases the direction of migration to coincide with the direction of the features.93 The most common geometry of engineered substrates involves parallel grooves; these bias migration to occur along an axis, but cannot result in true directed migration.94 Recently, asymmetric, but regular substrates have been created that are able to bias migration not to a bidirectional axis, but to a single direction.95,96

The importance of topographic cues in vivo is best illustrated in two systems: the cornea and cancer. In the former, alignment of the extracellular matrix is critical for cellular alignment, which determines transparency and is, therefore, paramount. As mentioned earlier, the topographic cues formed by the collagen fibrils in the cornea combine with magnetic fields to guide cell migration during corneal wound healing. Tumour cell migration is known to be preceded by modifications to the surrounding matrix that align it radially; this arrangement enhances the invasive capacities of the cells, which migrate preferentially along ECM fibres.100

2.4.4 Guidance Arising from Cell-Cell Interactions

While the guidance mechanisms mentioned above can be used to guide both single cells and cell collectives, cell-cell interactions are also known to affect the migratory behaviour of cells. One of the main examples, mentioned above, is the ability of leader cells (such as the tip cells in sprouting vessels) to pull along other cells to migrate.

Leader cells have been found to be critical in a number of migratory events in vivo. The role of tip cells in angiogenesis has been discussed above, and so will not be repeated here. Another example is the collective migration of the lateral line in zebrafish, which requires chemotactic guidance from SDF1. Lateral line migration has been shown to proceed correctly even when the majority of the cells are CXCR4-negative, as long as the leading cells express the receptor102,
which indicates a small proportion of the cells is sufficient to drive the migration of the whole group.

*In vitro* studies have also investigated the importance of leader cells to sheet migration and found that leader characteristics are granted by growth factor signalling\textsuperscript{103} and are dependent on RhoA activity\textsuperscript{104}. Consistent with this, others have found that increased cytoskeletal tension is likely to lead to leader cell formation\textsuperscript{105}. Leader cells are important for directional migration, and their absence may significantly retard migration in some cell types\textsuperscript{106}. This is likely due to an effect on coordination of directional migration\textsuperscript{103}: evidence has shown that all cells in a migrating sheet contribute to its motion\textsuperscript{53}, but that the migration of non-leader cells is random, while that of leader cells is directed\textsuperscript{103}.

The importance of force transmission across cell junctions in migrating collectives cannot be overstated: in 2D sheets, traction force measurements have found that force generation in each cell drives the migration of the whole sheet\textsuperscript{53,107}. Further, mechanical connections have been shown to be necessary for the propagation of coordinated motion in response to a scratch wound\textsuperscript{108}. However, we have also found that, in constrained environments, steric interactions between cells are enough to drive coordinate migration, even in the absence of cell-cell junctions\textsuperscript{94}.

Cell migration is, in reality, driven by combinations of these guidance mechanisms. Whether one of them dominates is likely dependent on context and cell type.

### 2.5 Collective Cell Migration *in vivo*

The early embryo is characterised by relentless change, but the progression from early zygote to fetus is tightly regimented. Most steps are critical, with errors leading to malformed organisms at best or embryonic lethalitys at worst. Many of these steps involve collective cell migration. The examples below do not constitute an extensive list.

In mammals, the migration of the anterior visceral endoderm (AVE) is one of the earliest crucial steps. The AVE restricts the formation of the primitive streak to the posterior side\textsuperscript{109,110}, which makes its positioning pivotal for patterning of the body plan. AVE cells differentiate at the distal tip of the egg cylinder around E5.0-5.5, and begin actively migrating at approximately E5.5-
5.75\textsuperscript{111}. After migrating approximately 100µm to where the epiblast meets the extraembryonic ectoderm (ExE) in 4-5 hours, they stop actively extending filopodia but continue rearranging passively as they are pushed laterally by migrating cells behind them. Differences in PCP signalling between the epiblast and the ExE are known to play a role in stopping the migration of the AVE at the junction of these two tissues. In \textit{Celsr} mutants, not only do the AVE cells spread over a much wider area, but fail to stop at the junction. Like with other morphogenetic processes, imaging AVE is complicated\textsuperscript{111}: The use of dyes is not recommended because it delays cell division, requiring the use of transgenic animals. The timing of the onset of migration is relatively variable, depending on when the mother’s vaginal plug is first detected. Because of culture conditions, not all embryos develop normally, and less than 50% of those that do provide sufficient cellular detail (though this has increased with improvements in imaging and image analysis advances\textsuperscript{112}).

One of the most common and important cell migration-related morphogenetic processes is angiogenesis, the main process by which blood vessels are formed from existing ones\textsuperscript{85,113-115}. Angiogenesis is critical during development because a complete vascular network is essential for survival\textsuperscript{116}, but continues to be important after birth because new vessels continue to be necessary as organisms grow and to provide nutrients and oxygen to new tissue in case of injury\textsuperscript{117}. New vessels are also central to the growth of many cancers, as tumours require increasing amounts of nutrients as they grow\textsuperscript{87}.
The migration of endothelial cells (EC) \textit{in vivo} is driven by leader (‘tip’) cells located at the front of the extending vessel. Signalling by VEGF renders all cells equally likely to become leaders; signalling through the Delta-Notch pathway (increased levels of Dll4) determine which cells become tip cells in the incipient sprout (Figure 2.4 from 118). Neighbouring cells then upregulate their Notch expression to reduce their expression of VEGF receptor (VEGFR) and become followers (‘stalk cells’)\textsuperscript{118}. However, the tip position is not fixed; shuffling occurs between tip and stalk cells often in the migratory process\textsuperscript{119}. Despite the requirement for growth-factor induced chemotaxis, the migration of EC in angiogenesis is also influenced by other factors, such as the extracellular matrix in a haptotactic manner (though this is uncertain), as well as mechanical signalling arising from fluid flow-induced shear stress (from blood flow)\textsuperscript{118}. Some evidence for haptotactic migration in combination with chemotaxis comes from \textit{in vitro} studies showing increased migration on a number of ECM molecules\textsuperscript{120} as well as increased expression of $\alpha_\text{v}\beta_5$, $\alpha_\text{v}\beta_1$, and $\alpha_\text{v}\beta_1$ integrins\textsuperscript{121}, which have all been shown to promote angiogenesis. The latter two are also associated with migration on fibronectin, and collagen, respectively.
An oft-cited example of collective cell migration in clusters is the migration of the border cells in the developing *Drosophila* egg chamber. The migration of border cells is initiated during stage 9 of oogenesis by Janus Kinase/signal transducers and activator of transcription (JAK/STAT) signalling from the polar cells, two differentiated epithelial cells located at the most anterior point of the chamber. This induces the delamination of the polar cells as well as the surrounding 6-8 cells from the epithelium in the form of a cluster with the polar cells at the centre. This cluster migrates in a directed fashion toward the posterior until it reaches the oocyte in stage 10. Border cell migration is an interesting model of collective migration for a number of reasons: the polar cells are known to be non-migratory; yet the cluster migrates effectively; the cells maintain their epithelial character throughout the migration; and the migration is directed by growth factor signalling. Additionally, unlike in other common models, the migration of the border cells occurs over other cells rather than ECM, and uses cadherin for traction rather than integrins. This invasive migration might share similarities with some cancer invasion processes; in fact, genetic analysis of *Drosophila* border cell migration has been used to guide research about ovarian cancer invasion. However, some of the advantages of the border cells as a model are also some of their disadvantages. While this model may reflect the invasion of some cancers, most cancers invade ECM-rich environments and use integrins for migration. Cluster migration is also relatively rare outside of cancer invasion. Further, other model systems have shown that many characteristics of collective cell migration are emergent, and so cannot be reproduced by smaller cell collectives.

Because collective cell migration is a hallmark of wound healing, wound healing models are also used often to study migration. The two main models for this purpose are skin and corneal wound healing, which are both accessible, can be imaged easily with existing tools, and involve relatively rapid migration. Skin biopsies and corneal injuries (eg. removal of the cornea, alkali burns, biopsies) are simple and can be monitored for healing by performing sequential imaging studies. However, despite the fact that these processes are relatively faster than other collective migration events in the body, they still have time lengths in the scale of days to weeks, which are incompatible with real-time imaging or cell tracking, making it difficult to understand individual cells dynamics during wound healing in vivo.

Studies of wound healing in the cornea have provided interesting evidence in the field of cell migration guidance: cell staining has shown that epithelial cells in wounded animals migrate in a
centripetal manner mandated by magnetic fields in the eye arising from small electrical currents\textsuperscript{133,134}. Additionally, others have found that the basement membrane upon which these cells migrate possesses nanotopographic features\textsuperscript{135} that may also guide migration\textsuperscript{136}. When both electrical and topographic cues are present and direct the cells in the same direction, increased directness is expected. However, when the cues are perpendicular to each other, corneal cells prioritize electrotaxis over topographic cues\textsuperscript{137}, indicating there may be a hierarchy of guidance mechanisms which have yet to be elucidated.

Many other morphogenetic, regenerative, and pathological events make use of collective migration, such as the formation of branched and/or tubular structures like the salivary gland\textsuperscript{138,139}, the trachea\textsuperscript{140}, the mammary gland\textsuperscript{24,60}, and the kidney\textsuperscript{141-143}; the migration of the lateral line\textsuperscript{144} and extension of the tailbud\textsuperscript{145} in zebrafish; the regeneration of peripheral nerves after injury\textsuperscript{146}; and the invasion of cancer cells into healthy tissues\textsuperscript{126-128,147}. However, despite these myriad examples, studying collective migration \textit{in vivo} is not ideal for many reasons: real-time image acquisition techniques are still limited to very early embryos or superficial structures; the long timescales associated with many collective migration processes do not easily lend themselves to timelapse imaging; and systematic manipulation of parameters is all but impossible in live animals. For this reason, \textit{in vitro} studies of collective cell migration are frequently preferred.

2.6 Studying Cell Migration and Cell Rearrangements \textit{in vitro}

The many difficulties associated with studying cell migration \textit{in vivo} have led researchers to develop a variety of \textit{in vitro} assays for single and collective cell migration, as well as for cell rearrangements.

2.6.1 Migration assays

The Boyden chamber assay is commonly used to determine the ability of cells to migrate. In this assay, cells are seeded on one side (usually the top) of a porous membrane that separates two media compartments, and the number of cells that traverse the membrane is determined at a later time point. The Boyden chamber assay was originally developed\textsuperscript{148} to determine whether cells respond to a chemoattractant by adding it to the bottom compartment; this creates a chemical gradient (albeit one of undetermined characteristics) across the membrane. The assay can also be
used to determine whether a compound increases random migration by adding the compound to both the top and bottom compartments\textsuperscript{149}. A variation of the Boyden chamber assay is used to study cell invasion by coating the filter with a layer of gel (often collagen or Matrigel)\textsuperscript{150}. As cells degrade the gel, they are able to pass through the pores, and can be counted as in the traditional Boyden assay. While these two assays are commonly used in migration experiments, they have a number of disadvantages: firstly, the proportion of cells that migrate across the membrane is typically several orders of magnitude below the number of cells seeded. Secondly, the dynamics of migration cannot be characterised since the assay only looks at population-wide behaviour and is not well-suited for temporal analysis. Lastly, the choice of membrane (particularly regarding pore size and, when dealing with adherent cells or the invasion assay, coatings\textsuperscript{151}), chemoattractant, and timing for this assay are non-trivial.

Early cell migration experiments often observed the migration behaviour of single cells on a 2D surface. These experiments are simple in that no specialized equipment is necessary and few cells are needed. However, many cell types migrate at very low rates when seeded sparsely (if the cells actually survive); if seeded at higher densities, the data becomes confounded by cell-cell collisions and intercellular signalling.

A more powerful yet still simple assay for studying cell migration is the scratch wound assay. This technique requires that cells be seeded as a confluent monolayer, which is then scratched to produce a cell-free “wound”. Originally developed to probe the effect of contact inhibition on cell proliferation, the scratch wound assay is now more commonly used to observe cell migration. Typically, the assay is used as an endpoint assay looking at population-wide behaviour\textsuperscript{152}, with the size of the cell-free area being measured at the start of the experiment and at the end to calculate the change, but timelapse imaging or individual cell analyses are also possible\textsuperscript{153}.

The scratch wound assay has been widely adopted because of its simplicity. It can be performed using only typical cell culture materials and analysed without specialized microscopes, software or equipment\textsuperscript{154}. It can be multiplexed to test the effects of cell-extracellular matrix interactions\textsuperscript{155,156}, drug testing\textsuperscript{157}, and gene modifications\textsuperscript{103} on cell migration on a large scale. It also has some similarities to \textit{in vivo} responses to endothelial\textsuperscript{158} and epithelial\textsuperscript{131} injury. However, the scratch wound assay also has disadvantages. The act of wounding the cell sheet releases
cellular contents and chemical damage signals, which combine with free space and the sudden release of tension in the sheet to create a complex signalling milieu which may affect cellular behaviour\textsuperscript{159,160}. It is also possible that the cells at the wound edge may be permeabilized\textsuperscript{159} as their intercellular junctions are destroyed, and hence be affected by being exposed to the cell culture medium\textsuperscript{161}. The action of mechanically scraping the cell sheet also alters and causes damage to the underlying ECM\textsuperscript{162,163}, and may not be ideal for cells which form very strong junctions, and are hence difficult to tear away from each other. Further, because of the inherent asymmetry in the setup, a chemotactic cellular response cannot be distinguished from a chemokinetic one\textsuperscript{154}. Moreover, the timing of a scratch wound assay is critical: in order to measure the effects of cell migration only (as opposed to cell proliferation), the endpoint must be significantly less than the time length for a population doubling\textsuperscript{153}, but must also allow cells to migrate enough to detect differences\textsuperscript{164}.

On the technical side, the scratch wound assay also requires considerations. For example, not all cell types migrate as a sheet. Calculating area changes when the sheet loses cohesion is typically meaningless considering cells spread differently in a sheet than when seeded sparsely. Analyzing single cell trajectories in these cases is potentially more appropriate, but requires timelapse imaging which is not always an option. The results of this assay are also heavily dependent on the initial density of the cell sheet\textsuperscript{165}. Another issue is that creating a straight, regular wound with limited cellular debris at the edge in a reproducible manner is not simple\textsuperscript{166}. Multiple protocols have suggested solutions for this issue\textsuperscript{154,165,167}, but experience is often the only way to make the assay fully reproducible.

To overcome some of the issues associated with the scratch wound assay, barrier or exclusion methods have been developed in which the cell-free area is created not by wounding the cell sheet, but rather by seeding cells around a barrier\textsuperscript{161,168}. After a monolayer has formed, the barrier is removed, with the cells moving to fill in the gap as in the scratch wound assay. This method avoids issues with cellular damage and wound shape described above. Similarly, cells can also be seeded in a colony-like configuration within a barrier\textsuperscript{105,136,169}. As that barrier is removed, the colony expands outward as cells migrate.

Exclusion-type assays are advantageous because, in general, the “wounds” created are better defined than those in the traditional scratch wound assay. In addition, by forgoing scraping of the
cell sheet, these types of assays avoid cell and matrix damage and so are able to focus exclusively on the effects of free space. There is some debate as to whether the placement of barriers affects any protein coatings on the cell culture substrate, but also some limited evidence that the matrix remains intact. Exclusion assays where the barriers are not pre-set allow for a variety of coatings to be applied to the culture substrate, making them particularly attractive for testing the effect of a variety of matrix proteins on cell migration; depending on the type of barrier used, some of these even permit the use of wet surfaces which may be preferable for certain proteins. However, these assays are not necessarily as straight-forward to perform as the scratch wound assay. One of the main concerns with solid barrier type assays is the fact that most of them require manual removal of the barriers, which is difficult to automate, and can result in cell damage at the wound edge. The placement of solid barriers can be disruptive to wet protein coatings as the liquid gets displaced as the barrier is put into place, and other types may not create a perfect seal against the substrate, allowing cells to penetrate underneath.

Some have suggested that, since these assays do not cause cell damage, they are perhaps more appropriate models for circumstances in which migration is not triggered by injury, such as cancer migration. An additional factor that is not considered for solid barrier assays is that the cells at the edge become aligned with the barrier, and lack junction proteins where they contact the barrier. This may affect the migratory behaviour of the cells in ways as yet unexplored.

Some of the most common barrier-type assay do not make use of solid barriers but instead take advantage of difference in surface tension between culture media and other fluids, such as air or oil. A cell suspension droplet is placed on a substrate delicately to ensure that the surface tension differences between the culture media and the surrounding environment are enough to prevent the droplet from dispersing. The cells in the droplet settle down on the area defined by the contact between the droplet and the substrate, forming a small colony. Once the cells have attached, the non-aqueous material can be removed if necessary; additional medium is added to ensure the cells have sufficient nutrients. These types of assays are even simpler to perform than the traditional scratch wound assay, making them very attractive. However, they are only suitable for cells that attach to the surface quickly because additional medium must be added in a short time frame to ensure the cells are not affected by lack of nutrients or oxygen. Further, because the colonies are relatively small, the cells must not be given to cell dispersion or...
experiments must be conducted shortly after seeding; otherwise, the colony will disappear as cells move away after the introduction of cell media. Lastly, these types of assays do not provide much control over the shape or quantity of cells that make up a colony, unlike other methods. Most importantly, it has been shown that some cell types require damage signals to trigger the onset of migration\textsuperscript{165}, so barrier-types assays are not always suitable for migration studies.

There are many other variants of wound-like assays that aim at mitigating some of the issues with the scratch wound assay. These include creating gaps in cell sheets using electricity\textsuperscript{174} and enzymatic\textsuperscript{175} or chemical\textsuperscript{176} cell removal, which can involve microfluidic devices. The placement of polydimethylsiloxane (PDMS) stamps on top of intact cell sheets to cause cell death has also been use to study cell migration in the presence of cell debris\textsuperscript{177}. However, use of these variants is rare because they require equipment that is not found in all biology labs as well as additional expertise to be performed correctly.

In addition to the methods above, other techniques for “woundless” wound healing and colony expansion make use of micropatterning techniques. By patterning a non-permissive surface with extracellular matrix proteins or using specialized chemistry to create sheets in defined geometries, which are then released by making the whole surface permissive, it is possible to avoid some of the concerns that arise in barrier-type assays, such as disruption of the matrix by barriers. Alignment of cells at the edge of the pattern, however, remains\textsuperscript{178}. Micropatterning requires more expertise and equipment than other assays, but can also offer more flexibility in terms of geometries\textsuperscript{179–182}.

While micropatterning techniques can be used for colony expansion, allowing cells to migrate within patterns is also helpful to understand cell behaviour in constrained spaces. These types of assays have been used to study symmetry breaking, chirality\textsuperscript{181}, and the emergence of collective behaviours in migration\textsuperscript{182,183}. However, despite the fact that these types of assays are particularly useful in helping to simplify the cues that cells are exposed to in order to answer complex questions, they are seldom relevant to \textit{in vivo} situations. They also have many of the drawbacks of barrier-type assays, such as the lack of damage cues, while involving much more difficult protocols.

The study of cells within constrained environments can also be performed without the use of micropatterning. Seeding confluent sheets within wells is much simpler than micropatterning
specific shapes, and can provide similar results (for example, in regards to cell rotation). Seeding cells within physical enclosures has some disadvantages compared to micropatterning: cell alignment at the edge and, potentially, edge effects propagating into the sheet; microfabrication of small enclosures and cell seeding within those is not simple; and less flexibility in terms of geometries. On the other hand, existing plates and other cell culture equipment can be used with no modifications.

Most of the assays above can be augmented by the addition of a variety of signals. Some examples include topographic cues; mechanical forces, such as shear stress; substrate stiffness; and electrical fields. Additionally, while numerous 3D assays have also been developed to characterise 3D migration and invasion processes, they are outside the scope of this thesis.

However, it is worth noting that despite the many advantages of in vitro studies, these types of assays also carry numerous disadvantages, with the most impactful of these perhaps being the lack of appropriate environmental cues: cells in the body are inevitably and relentlessly exposed to cues from other cell types, their underlying substrate, the immune and endocrine systems, and many other signals. This complexity is near impossible to replicate in vitro, which is a major drawback considering the evidence that all of these cues may affect migratory behaviour. This is heightened by the fact that most in vitro assays are designed to analyse 2D behaviour, which while appropriate for some cell types, is often not representative of the true geometry experienced by the cells in vivo. Further to this point, in vitro assays typically use a single cell type and are performed on polystyrene or glass surfaces; this ignores cell-cell interactions and the much less stiff characteristics of the surfaces on which cells typically migrate within the body.

2.6.2 Preferred Cell Types for Studying Cell Migration

As in all other studies, experiments that study changes in cell migration due to disease, mutations or drugs are preferably conducted using a cell type that is as similar as possible as cells that would be affected in the body. However, this is not always practical: primary cells might be difficult to obtain, maintain, or work with in migration assays, in which case a model cell line may be a better choice. Model lines and/or easily accessible primary cell types are also preferred
when studies focus not on a particular condition, but seek to understand general principles for how cells migrate, as this is more likely to avoid cell-specific phenomena from arising.

### 2.6.2.1 Mesenchymal Cells

The earliest studies of cell migration were conducted by Abercrombie and Heaysman in the early 1950s\(^64\), and examined how chick heart fibroblasts dispersed from an explant. Fibroblasts continued to feature heavily in Abercrombie and Heaysman’s work, and were used to lay the foundations of what is recognized today as the multi-step lamellipodia-dependent process that underlies the migration of most cells\(^{187-190}\). Because fibroblast migration has been well-defined\(^39\), and in particular because the process of lamellipodial extension\(^{191,192}\), adhesion\(^{193-195}\), cell body contraction/force generation\(^{196,197}\) and rear withdrawal\(^{198,199}\) has been thoroughly characterised in fibroblasts\(^{200,201}\), they continue to be used regularly for migration studies. In most circumstances, fibroblasts migrate rapidly enough that their progression is easy to observe without losing information about their individual steps, unlike in faster cells\(^49\). Fibroblasts primarily migrate as single cells, which makes them ideal for studies such as those conducted by Abercrombie and Heaysman in 1970\(^{188-190}\) which focus on individual cell behaviours without considering the effect of cell-cell interactions. Fibroblasts are also advantageous for their relevance to in vivo situations, as they are commonly involved in in vivo wound healing\(^{202-205}\) and in cancer invasion\(^{206-209}\). However, fibroblasts are not appropriate for collective migration studies because they do not maintain strong cell-cell junctions nor migrate collectively under normal conditions. Though fibroblasts seeded at high densities can behave similarly to other cells types, migrating in ways that suggest supracellular coordination, this is likely to be due primarily to steric interactions between individual cells rather than other types of cell communication\(^{210}\). In wound healing assays, most (but not all. For example, L1 fibroblasts do not scatter significantly\(^{211}\), fibroblasts respond by scattering and are not able to migrate as a coherent sheet\(^{212,213}\).

Another mesenchymal cell type that is commonly used to study cell migration is neural crest (NC) cells. The coordinated migration of these cells is a well-known critical process during development, and studies of their interactions in vivo and in vitro have provided valuable information with regards to how coordinated chemotaxis\(^{214,215}\), contact inhibition of locomotion\(^{69,216}\), and directional guidance\(^{217-219}\) interact to accomplish very precise, complex directed migration. NC cells are attractive for migration studies because their migratory failures
result in severe developmental defects. They also provide some of the best examples of conserved migratory behaviours across species, with data arising from multiple model organisms including *Xenopus*\textsuperscript{220}, zebrafish\textsuperscript{221}, chicks\textsuperscript{222} and mice\textsuperscript{223}, which increases scientists’ ability to understand the essential factors for NC migration to proceed.

A major issue with NC migration research is one of language: because NC migration involves the coordinated migration of many cells, researchers often refer to it as a collective cell migration process. Unfortunately, this conflates this mesenchymal cell research with research focused on the migration of cells of epithelial origin which maintain strong cell-cell junctions. Attempts to separate the two have received some pushback\textsuperscript{224}, partly owing to the rising popularity of collective cell migration as a research topic, but also because this type of migration may involve coordinating mechanisms that are also present in epithelial cells. Similar issues arose when single cell migration was a nascent topic\textsuperscript{225}, and unifying language may be necessary to achieve better understanding of the commonalities and differences between these two behaviours.

### 2.6.2.2 Epithelial Cells

Keratinocytes are a popular cell type in collective cell migration studies because of their well-documented migratory behavior in skin wound healing\textsuperscript{226,227} and their role in eyelid fusion in the developing embryo\textsuperscript{228-231}. They’re also exceedingly easy to procure as primary cells from a variety of sources: fish scales\textsuperscript{184}, human foreskin from circumcision procedures\textsuperscript{232} or skin biopsies\textsuperscript{233}, for example. Some keratinocytes are strongly migratory, moving at relatively high speeds compared to other cell types, and increasing their persistence and directed movement when migrating collectively\textsuperscript{234}. However, most human keratinocytes are only moderately migratory in the absence of growth factors; they migrate much more effectively when one or more growth factors are added\textsuperscript{235}.

An epithelial cell type that has been used specifically for studying the interaction of migrating cells with their underlying substrate are Chinese Hamster Ovary (CHO) cells and their variants. Some of these cells are particularly well suited because they are deficient in a number of integrin subunits\textsuperscript{236}, which makes them ideal for exogenous expression of any integrin subunits of interest. CHO cells were used to generate one of the most common models\textsuperscript{237} for the relationship between cell speed and adhesion between a cell and the substrate: cell speed has a biphasic relationship with substrate adhesion strength and is maximal at an intermediate value of cell-
substrate adhesiveness, because at low values, adhesiveness is insufficient to balance cell body contractility effectively, while at high values, dissolution of adhesion is difficult to achieve\textsuperscript{52,238,239}. However, CHO cells are rarely used any longer, probably because of the limited integrin expression profiles that made them useful in the first place.

Another epithelial family of interest is the retinal pigmented epithelium (RPE), because of their involvement in healing eye injury\textsuperscript{240} and in retinopathies\textsuperscript{241}. Given this, many studies have focused on the directed migration of these cells, which have led to interesting results about the establishment, maintenance and propagation of directionality signals. For example, the persistent migration of RPE-1 was found to be abrogated when microtubule polymerisation is disrupted, suggesting a novel role for microtubules in the maintenance of cell polarity\textsuperscript{242}. Using these cells, others have shown that directional persistence is mediated at least in part by a stable and strongly attached cell rear; the detachment of the trailing tail of migrating cells is, in fact, responsible for changes in direction\textsuperscript{245}. In addition to studies about the internal mechanisms that govern intrinsic cell directionality, these cells are often used for research on the effect of external mechanisms on biased cell migration. We have published on the propagation of topographical cues through confluent sheets and its effects on retinal pigmented epithelial cell migration, and have shown that because the main mechanism for propagation is not junctional communication but mechanical interactions, this work is likely to extend to other epithelial and constrained mesenchymal cells\textsuperscript{210}. We have also shown that the migration of ARPE-19 cells in scratch wound healing is characterised by highly coordinated cell behaviour which greatly exceeds that within unwounded sheets\textsuperscript{244}. Electric fields have also been shown to play a role in directed RPE migration\textsuperscript{245}.

One of the most common cell types used in collective cell migration studies are Madin-Darby Canine Kidney (MDCK) cells. MDCK cells are used broadly in biology as an epithelial cell model\textsuperscript{246} because they seem to embody the main epithelial characteristics: they polarize well\textsuperscript{247}, resulting in clear basolateral and apical\textsuperscript{248} membrane domains defined by stable tight\textsuperscript{249} and adherens\textsuperscript{250} junctional complexes, which allows them to control sheet permeability\textsuperscript{251}. Renal cells are ideal for collective migration studies because kidney morphogenesis is driven by collective migration processes, so understanding the principles governing the collective migration of kidney-derived cells in \textit{in vitro} can provide useful guidance to better design experiments related to \textit{in vivo} kidney development. Unfortunately, as highlighted in \textsuperscript{246}, different
strains of MDCK are highly heterogeneous, which coupled with poor reporting of strain type makes comparison between studies difficult. That being said, some of more interesting observations about the mechanics and biophysics of collective cell migration in the last 15 years come from MDCK experiments, and have received more or less confirmation from other cell types, suggesting a general applicability to epithelial cell types. MDCK do not require a damage signal to migrate\textsuperscript{161}, but only do so after reaching a certain cell density threshold\textsuperscript{252}; however, others have shown that MDCK migration speed decreases as cell density increases\textsuperscript{253,254}. The differences may arise because the studies used different assays (scratch wound versus tracking in constrained confluent sheets), or may reflect that, after reaching confluence, speed increases in a small density range before decreasing. That these cells cannot migrate unless sufficiently dense might be a consequence of the fact that migration of MDCK is highly cooperative\textsuperscript{254}. studies have found that not only do MDCK cells extend “cryptic lamellipodia” several rows back from a scratch wound\textsuperscript{14}, but also that the traction forces that arise are heterogeneous\textsuperscript{53,255}, with “tug-of-war”-like behaviour occurring locally within the sheet\textsuperscript{130}. This heterogeneity is an important feature of collectively migrating sheets, and has been replicated in other cell types\textsuperscript{53}. Despite this cooperativity, MDCK migration is also known to depend on leader cells\textsuperscript{256}, the absence of which severely impairs migration\textsuperscript{106}.

In addition to the forces of collective cell migration, MDCK have been used to study the dynamics thereof and have provided evidence that scratch wound healing depends on waves of MAPK\textsuperscript{160} and ERK activation\textsuperscript{257} that propagate over time from the edge of the wound toward the back of the sheet. Others have also found that the dynamics of migration of MDCK cells share remarkable similarities with that of the motion within colloidal glasses (or supercooled fluids)\textsuperscript{258}. One of the main disadvantages of MDCK cells is their canine origin. Antibodies are more commonly made for mice/human tissues, and finding antibodies against rare proteins may be difficult. This, combined with the identity issues associated with the multiple strains mentioned above, may make MDCK cells unsuitable for a large variety of experimental avenues.

2.6.2.3 Endothelial cells

Because angiogenesis is perhaps the most common collective cell migration process and is highly relevant for cancer therapies, EC are also very common in collective migration research. Collective migration research in EC involves both 2D and 3D migration. While 3D migration is
beyond the scope of this thesis, significant insights into general collective migratory behaviours have been gained from 3D processes, particularly regarding the factors that determine leader identity in migrating collectives\textsuperscript{101}. One of the most interesting insights from 3D endothelial sprouting is that the leader position is not permanent, and instead is often re-evaluated and taken up by other cells\textsuperscript{119}; this constant exchange contrasts with epithelial cells, in which removal of the leading cell appears to hinder migration\textsuperscript{106}, suggesting leader identity is less plastic in migrating epithelium. Endothelial monolayers are also important because they have been used to gather critical information regarding cell steering and the mechanisms that coordinate collective cell migration. Like MDCK\textsuperscript{258}, EC are known to move within constrained environments in ways that are locally, but not globally coordinated\textsuperscript{259}, forming streams. This streaming behaviour has been recognized as an emergent property of collectively migrating cells, and is thought to arise from mechanical interactions due to cell incompressibility beyond a certain range\textsuperscript{94,130,260}.

However, other steering behaviours are also known to be important. An oft-cited study used a scratch wound assay to screen numerous siRNA constructs for migration effects. Hits were then sorted depending on whether they affected cell proliferation (which by virtue of occupying additional space forces cell motion and may in fact be the reason for observed motion within confluent sheets\textsuperscript{258}), directed migration, individual cell migration, or cell-cell coordination. The results suggested that the latter three are the main modules that control endothelial sheet migration, and potentially the coordinated migration of other cell types and/or other configurations\textsuperscript{103}. Further work by these authors found that, while a variety of genes affect cell migration, the behaviour of the cells can be predicted by a kinematics-based model that takes into consideration the cells’ adhesive, cohesive, and repulsive properties only, indicating that the genes found earlier may in fact change the mechanical and adhesive properties of the cells\textsuperscript{261}. The dependence of directed cell migration on stress transmission through junctional complexes mentioned earlier for MDCK has also been demonstrated in EC, further reinforcing the idea that mechanical interactions are critical in collective cell migration\textsuperscript{53}.

The fact that many different cell types have been demonstrated similar migratory behaviours lends credence to the idea that universal mechanisms exist that govern collective cell migration. However, depending on the study, certain cell types may be more appropriate than others, whether that be because of increased \textit{in vivo} relevance, ease of acquisition or manipulation, or
convenience/better fit with other reagents. Researchers should keep the differences between these cell types in mind when selecting the most appropriate for their own experiments.

2.6.3 Assays to Understand Cell Migration Dynamics

One of the most powerful techniques in cell migration is the tracking of cells in two dimensions. Pioneered by Abercrombie and Heaysman in 1953\textsuperscript{64} to understand the effects of cell-cell contact on the migration of fibroblasts from chick heart explants, this simple technique involves noting the position and changes thereof of one (or more) cell over a relatively long time period. From these positional changes, information can be gathered as to migration speed, turn rates and persistence. Though originally performed manually by comparing individual frames\textsuperscript{64} or by projecting film on to a screen and marking individual positions over time\textsuperscript{262}, this technique is now almost exclusively automated; cell tracking has greatly benefitted from advances in image analysis\textsuperscript{263} and computing that allow for precise and accurate tracking of thousands of objects over long periods of time. Cell tracking has the advantage of providing fine-grained information about both the spatial and temporal dynamics of cell migration. More importantly, its focus on single cell behaviour makes it possible to understand the rise of emergent behaviours that are not necessarily captured in other techniques.

Another technique commonly used to collect cell migration information is Particle Image Velocimetry (PIV). PIV was originally developed for fluid dynamics studies, and used small particles to generate fluid velocity vector fields that could be used to understand turbulent flow patterns\textsuperscript{264}. This type of analysis is helpful in understanding the patterns of particle/cell movement, and is less time-intensive than single-cell tracking, but cannot provide information at the single cell level. However, a comparison of both techniques has shown that, on a global scale, the results obtained are similar\textsuperscript{254}, which makes this technique attractive when resources are limited and detailed single-cell information is not required. PIV also preferable for 3D migration studies, so long as imaging of the objects is possible because of its lower use of resources when compared to single object tracking.

A variation on PIV, called Monolayer Stress Microscopy (MSM), has been developed for measurement of intercellular stresses\textsuperscript{265}. In MSM, cells are seeded on a deformable substrate that contains fluorescent beads. PIV is then used to track the motion of the beads in the substrate as the cells move and deform the substrate below them. This information, combined with material
properties of the substrate can be used to calculate the forces exerted by the cells on the substrate as they migrate. Performing force balances on the cells then provides the intercellular stresses.

Though these are the best methods in use currently for the study of collective cell migration dynamics, they are often complimented with high resolution timelapse imaging studies to understand subcellular changes. Timelapse studies of scratch wound healing assays are also used occasionally to understand wound closure dynamics.

2.6.4 Metrics to Understand Cell Migration Dynamics

For years, the migration of cells has been described as a persistent random walk (even though the behaviour of many cell types does not support that description)\textsuperscript{266}. In the classic random walk description, the movement of an object is taken in steps, with the direction, length, and timing of subsequent steps being randomly chosen and having no dependence on previous movements; for cells, the model has been changed by increasing the probability that the direction of each step will match that of previous steps. Other modifications can be made to include directional guidance mechanisms\textsuperscript{267}.

A random walk model describes diffusive processes, and its most common metric is that of mean square displacement (MSD). In perfectly diffusive processes, MSD increases linearly as time grows\textsuperscript{268}. In contrast, when the movement of the object depends on previous trajectories, and is ballistic, MSD has a quadratic relationship with time. MSD is frequently reported in cell migration articles, but given the mounting evidence that many cells types do not move in a random walk fashion, it has become less common in recent years.

More common metrics attempt to describe the behaviour of individual cells and of the population as a whole (see chapter 5 for more discussion on these metrics and examples thereof): distributions of cell speed (typically obtained by measuring either the distance moved between two time frames and dividing by the time between them – instantaneous speed – or by adding the total distance travelled through all time frames and dividing by total time – average speed), net velocity (the net displacement between the first and last time frames divided by total time), and persistence/directionality ratio (the ratio of speed and velocity) are common. Because the distribution of speeds normally has high kurtosis and skewness (as only a few cells migrate
rapidly), medians of these values are also appropriate to report. When directed migration is under study, angular distributions are also common.

The emergent properties of collective cell migration also necessitate the use of metrics that can describe these collective behaviours. The most commonly reported is correlation length. Correlation length is a metric derived from statistical mechanics that can be used to describe the order of a multiparticle system. Correlation length is calculated by taking the dot product of vectors describing the motion of each cell/field, averaging the results, and finding when the value decreases to zero. Because correlation length is averaged along all directions, it fails to take into consideration that, unlike particle motion, cell migration is polarized, and hence more likely to be correlated in some directions than in others. In order to address this, we have created a new metric, which we named stream length (see chapter 3). Stream length focuses on movement parallel to the cell’s on either side of it.

In physics, an order parameter is a metric that can be used to describe phase transitions in a system. For example, the order parameter of the liquid-gas transition is density (or rather density minus density at the critical point). This value is zero for the liquid phase, but jumps discontinuously at the liquid-gas transition. Order parameters are useful in multi-particle systems because they can describe changes in system behaviour easily. For this reason, in addition to the standard metrics described above, because migration studies often focus on different aspects of migratory behaviour, question-specific metrics are typically necessary. For example, in chapter 6, where we sought to understand how signals propagate across a migrating sheet, we designed a new metric, propagation length. Propagation length measures how far from a grooved substrate cells migrate in a biased manner; because this bias is bidirectional, we calculated the square of the cosine between the direction of the grooves and each cell’s direction of migration. We then examined how this value decayed as a function of distance from the grooved substrate, and set propagation length to correspond to when the average migration direction was more than 25° different than the direction of the grooves. Others have used metrics such as timed-average direction of motion, the strength of cell-substrate adhesion, and the cosine of the angle of migration with respect to guiding features as order parameters to answer specific questions.
2.7 Conclusions

Cell migration is a complicated process that involves the integration of signals at the molecular, cellular, and supracellular levels. Understanding how changes in these parameters affect cell motion is important and so numerous experimental techniques have been developed to study and measure cell migration. However, while significant attention has been paid to how changes at the molecular scale affect migration at the cellular and supracellular levels, little is known about how combinations of factors (for example, changes at the molecular levels combined with supracellular guidance mechanisms) affect behaviour. Understanding how multifactorial changes affect cell behaviour is important because of how critical cell migration is to development, regeneration and disease. This work proposes new tools to study how guidance cues combine to effect cell migration behaviour in order to answer these important questions.

2.8 References


Chapter 3
An Algorithm to Quantify Correlated Collective Cell Migration Behavior

3.1 Commentary

This work underwent peer review and was published in Biotechniques in February 2013 (Slater, B., Londono, C. & McGuigan, A. P. An algorithm to quantify correlated collective cell migration behaviour. *BioTechniques* 54, 87-92 (2013)). Author Contributions: CL and APM devised metric. BS created the algorithm for calculations and wrote all code. CL provided all experimental data. BS wrote the manuscript with input from CL and APM. BS, CL and APM analysed the data.

Though major advances have occurred in the field of image acquisition and analysis for collective cell migration, the analytical methods for quantifying that data have not kept up with those advances. Most of the current metrics used to analyse migration experiments are simple descriptors of motion, which have been in use to describe cell motion since the 1950s. Because until recently, we were unable to acquire and process the large amounts of data generated in collective cell migration experiments, few metrics have been generated to deal with this phenomenon. Current approaches, such as correlation length, borrow from statistical mechanics analyses, but fail to take advantage of the ability to track migration on an object-by-object basis in collective cell migration.

We describe here an algorithm to measure streaming, which refers to the tendency of groups of cells to migrate together. Because physical constraints make it likely for cells located in front and behind one another to migrate in the same direction, the algorithm examines only cells which are located to the sides of the cell of interest.

This work offered one of few metrics currently existing that was designed specifically to describe collective cell migration and that took full advantage of the ability to track individual objects in collective cell migration when compared to other multiparticle systems.
3.2 Abstract

Collective cell migration is an important process that determines cell re-organization in a number of biological events such as development and regeneration. Random cell re-organization within a confluent monolayer is a popular *in vitro* model system for understanding the mechanisms that underlie coordination between neighboring cells during collective motion. Here, we describe a simple automated C++ algorithm to quantify the width of streams of correlated cells moving within monolayers. Our method is efficient and allows analysis of thousands of cells in under a minute; analysis of large data sets is therefore possible without limitations due to computational time, a common analysis bottleneck. Furthermore, our method allows characterization of the variability in correlated stream widths among a cell monolayer. We quantify stream width in the human retinal epithelial cell line ARPE-19 and the fibroblast cell line BJ, and find that for both cell types stream widths within the monolayer vary in size significantly with a peak width of 40 µm, corresponding to a width of approximately two cells. Our algorithm provides a novel analytical tool to quantify and analyse correlated cell movement in confluent sheets at a population level and to assess factors that impact coordinated collective cell migration.

3.3 Introduction

Collective cell migration is critical in numerous biological processes such as embryo morphogenesis\(^1,2\), angiogenesis\(^3\) and wound healing\(^4\). It also plays a central role in pathological situations such as metastasis, allowing some tumour cells to invade surrounding tissues\(^5\). However, despite the importance of collective cell migration, how cell coordination occurs during group migration is not well understood\(^6\). A better understanding of the underlying behaviours during collective cell migration will provide insight into embryo development and tissue re-organization during regeneration and disease. Furthermore, the ability to control collective migration will provide novel tools for engineering tissues with reproducible structures.

There are a number of *in vivo*\(^7-9\) and *in vitro* model systems\(^5,10-12\) used to study collective cell migration, both directed migration and random migration. Two common *in vitro* models are confluent sheets of cells\(^13\) with or without a wound intended to induce directed migration. These simple 2D model systems are powerful because they allow tracking of individual cells within the sheet and careful characterization of the group behaviour\(^14\) and cell-cell coordination\(^15\) that arises from cell-cell interactions between neighboring cells. Specifically, cells within the sheet
randomly exchange places with their neighbors resulting in a net displacement from their original location\textsuperscript{16} and the formation of randomly oriented cellular “streams” characterised by correlated cell motion\textsuperscript{16,17}. When a wound is present, the streams at the wound edge become oriented and directed cell movement into the free space occurs to close the wound. Within a cellular stream during both random and directed migration, motion is coordinated between neighboring cells (i.e. across the width of the stream) and between cells moving behind and ahead of each other (i.e. along the length of the stream). While cell coordination along the stream length could arise passively from the creation of free space behind a cell, coordination across the width of the stream requires more complex cellular interactions. Therefore, understanding the behaviour of cells along the width of a stream can potentially provide more insight into the mechanisms of cell-cell coordination during sheet re-organization. Furthermore, quantifying stream width is potentially useful for designing culture surfaces\textsuperscript{18} with adhesive or topographic features (unpublished data) that allow manipulation of this streaming behaviour, where feature dimensions must be on the scale of the stream width\textsuperscript{18}.

A common method for quantifying cell coordination is the velocity correlation function\textsuperscript{16,19-21} which evaluates the overall correlation of cell trajectories as a function of distance from a cell using the equation:

$$V(r) = \frac{1}{N} \sum_{i=1}^{N} \left( \frac{1}{n} \sum_{j=1}^{n} \frac{(\mathbf{v}_i \cdot \mathbf{v}_j)}{|\mathbf{v}_i||\mathbf{v}_j|} \right), \text{[Equation 1]}$$

where $N$ refers to the total number of objects being analysed, $n$ is the number of neighboring objects within a radius, $r$, from the current object $i$, and $j$ refers to all other objects within radius $r$. By averaging all dot product values for all cell pairs, the function provides an overall description of neighboring cell correlation as a function of distance from a cell. When the velocity correlation function falls to approximately 0 (or $\theta_{\text{average}} = \pi/2$), cells are no longer considered correlated. The radius at which this occurs provides a measurement of the correlated domain (a combination of stream width and length). A major drawback of the velocity correlation function is that it is inherently biased by the behaviour of the cells both in front and behind the cell of interest. For example, when streams extend over large distances compared to the stream width, such as in fast moving cell types, the velocity correlation function reports positive correlation over a larger distance than the stream width because correlation along the
direction of motion offsets the diminishing correlation in the axis perpendicular to the direction of motion. Furthermore, cell behaviour within a confluent sheet is highly variable and since the velocity correlation function is often presented as the average for all cells, information about the variation in behaviour among the population is lost. Here, we describe an algorithm to explicitly quantify the width of cellular streams and the variability in the width of stream within a confluent cell sheet, with or without a wound.

3.4 Materials and Methods

*Cell culture*

We conducted experiments using human retinal epithelial cells (ARPE-19) (ATTC, Manassas, VA, USA) and human foreskin fibroblast cells (BJ) (ATTC). ARPE-19 were grown in Dulbecco’s modified Eagle’s medium/nutrient F-12 (DMEM/F-12) (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (VWR International, Mississauga, ON, Canada) and 1% Penicillin/Streptomycin (VWR). BJ were grown in DMEM (ATCC) supplemented with 10% FBS and 1% Pen/Strep. All cells were maintained in a humidified atmosphere at 37°C and 5% CO₂. For some experiments we over-expressed GFP-N-Cadherin in ARPE19 cells using a lentivirus over-expression vector, followed by sorting of the cells by flow cytometry to ensure a homogenous population.
Live cell imaging and tracking

We seeded 64,000 cells/cm² in a custom-made 96-well PDMS flat bottom plate to generate confluent cell sheets (Figure 3.1A). For wound healing studies, 90,000 cells/cm² were seeded in a 24-well TCPS plate coated with 314µg/mL PureCol (Advanced Biomatrix, San Diego, CA, USA) for 1 hour prior to seeding and wounds were made using a P10 tip. We tracked cells a day later using a previously established protocol\(^{15,22}\). Briefly, cells were stained with 500ng/mL Hoechst 33342 (Invitrogen) in culture medium for 30min, followed by a PBS wash and addition of culture medium. An ImageXpress Micro high-content screening microscope with a live-cell imaging module (Molecular Devices, Sunnyvale, CA, USA) was used to take images of the cell
nuclei every 20 minutes for 8 hours for tracking in confluent sheets (Figure I.1 and movie S3.1 – See Appendix I) and for 23 hours for wound healing (Figure I.2 and movie S3.2 – See Appendix I). Throughout the experiment, cells were maintained in a humidified atmosphere at 37°C and 5% CO₂. Positional tracking of cells in each well was performed using the Multi-Dimensional Motion Analysis application module in the MetaXpress software package (Molecular Devices). This tracking algorithm uses nuclear shape and fluorescent intensity measurements to track the position of individual cells (Figure I.1 and I.2 – See Appendix I)²³,²⁴.

Stream visualization and quantification

To assess the width of cellular streams, we input the text files generated from the MetaXpress tracking algorithm into our analysis program. The text files contain information for individual cells over a series of time frames including the cells' assigned object numbers, their positions and radii from a designated origin, and the angles that they are moving at relative to the previous time frame. These variables are specifically used in the correlation algorithm to evaluate the correlated stream widths. While we used text files generated by MetaXpress, any method of cell tracking that gathers this coordinate data, including manual tracking, could be used with our algorithm with minor modifications to the code. For comparison, we also calculated the velocity correlation function using equation 1¹⁶,¹⁹-²¹. The resulting average of the velocity correlation function falls between zero and one (zero means no correlation between cell streams and one means that all streams are moving in the same direction)¹⁶,¹⁸,²⁵.

The MetaXpress tracking algorithm also outputs an image file that allows the visualization of the moving streams (refer to Figure 3.1B), but the color coding is random. To better visualize the coordination between cells in the stream, we wrote a MATLAB (The MathWorks, Natick, MA, USA) program that specifically colors the moving cell streams based on the direction of motion from the text files (refer to Figure 3.1C and D). Using these latter images, we estimated the accuracy of our algorithm by comparing reported stream widths for specific cells with manual measurements for those cells from the angle-based colored images.
3.5 Algorithm Design

Figure 3.2 – Schematic of analysis algorithm: (A) and (B) depict a step by step explanation as to how the algorithm functions. (A) The cell in the center is being compared to neighboring cells within the ring of thickness 20µm ($r_{in}=0$ and $r_{out}=20$ µm). Since the neighboring cells are moving within 10°, the algorithm extends the assessment radius to evaluate neighboring cells within the next 20µm ring in B. In (B) the cells in this new ring ($r_{in}=20$ µm and $r_{out}=40$ µm) are not moving within 10° of the current cell, so the algorithm will stop and return the distance of the previous radius where correlated cells were observed.

An overview of the algorithm is shown in Figure 3.2. To evaluate the widths of the moving cell streams, the algorithm compares the angle of displacement for a given cell (the comparison cell) at a given time point with the angles of displacements of certain surrounding cells at that same time point. Specifically, the algorithm compares the angle of displacement of surrounding cells located within a 140° arc perpendicular (above and below) to the angle of displacement of the comparison cell (Figure 3.2A). We did this to avoid overestimating stream width due to correlated movement along the stream length. For each surrounding cell, the algorithm designates the cell as correlated with the comparison cell if it is moving within 10° relative to the
direction of the comparison cell. This threshold angle can be changed if desired. Only objects in
the same time frame are compared because objects moving in the same direction as the
comparison cell at earlier or later time frames are not necessarily part of the same cellular
stream.

The algorithm assesses the correlation of surrounding cells at progressively increasing distances
from the comparison cell, and defines stream width as the distance at which correlation is lost.
Specifically, the algorithm examines the cells in the defined arcs that are within a ring of 20µm
thickness (r_in to r_out in Figure 3.2) from the comparison cell, starting at an inner radius of 0µm
(Figure 3.2A). This ring thickness was chosen because ARPE-19 and BJ cells have widths that
are within a 20 to 30µm range (25.4±6.4µm and 25.7±7.4µm, respectively); this number should
be altered if larger or smaller cell types are being assessed. Each successive ring is considered to
be correlated if the number of correlated cells is greater or equal to the number of uncorrelated
cells in either one or both arcs of the ring. If the algorithm detects the cells in a particular radius
are correlated, the algorithm expands the inner radii by a further 20µm (Figure 3.2B) and
compares the number of uncorrelated and correlated cells in arcs in the new ring region. This
comparison operation is continued until a ring is reached where the number of uncorrelated cells
is greater than the number of correlated cells (Figure 3.2B) in both 140° arc regions of that ring.
The inner radius of this ring is defined as the stream width for that object (comparison cell) for
that time frame. Since each 140° arc is defined separately, if one or both of the arcs do not
contain any cells in a given time frame, the inner radius is increased until new objects are found.
If objects are not found within 100µm, the arc is declared done and the inner radius where cells
were last previously detected is logged as the stream width. This component of the algorithm is
important in situations where cells are more spread out, at lower cell densities, at the boundaries
of the image, or if a wound is present. To account for situations where the comparison cell being
analysed is in the center of a stream, the radius is doubled if both arcs finish at the same time.
For a given comparison cell, the stream widths are analysed for all of the time frames and the
average radius is calculated and recorded as the stream width. This is then repeated for all cells
in the image. The algorithm therefore outputs a distribution of stream widths (one for each cell in
the image).

Our algorithm assesses the average stream width for a given cell at only selected time frames
(frames 1, 4, 8, 12, 16, 20 and 24). This decision was made because examining every frame
resulted in odd computations due to any random “wiggle” effects from non-straight movement of the cells. By eliminating frames, the cell movement was approximated to be more linear (see increase in straightness of path in skipped frame image compared to non-skipped frame image in Figure 3.1C and D). Also, the 140° arcs are subdivided into two halves (four sections total). This prevents the algorithm from terminating early in cases where rings contain neighboring streams moving in different orientation. This was not an issue at small assessment radii but became a problem after the size of the arcs was increased to find more objects in the same frame.
3.6 Results and Discussion

**Figure 3.3 - Quantification of stream width:** (A) Histogram showing the percentage of cells that occupy streams containing a given number of cells. (B) Histogram showing the percentage of cells that occupy streams of a given width. The peak in B is less apparent than in A because the algorithm averages up to the nearest whole cell number resulting in smoothing of the curve. (C) and (D) are plots of the average velocity correlation as a function of distance for ARPE-19 and BJ cells, respectively. (E) and (F) are plots showing the distributions of stream widths measured from the velocity correlation function data for ARPE-19 and BJ, respectively. (G) Histogram of the distribution of stream widths of ARPE-19 cells in the wound healing assay compared to a confluent sheet. (H) Histogram of the distribution of ARPE-19 cells expressing GFP-N-cadherin compared to WT ARPE-19 cells.
As expected, our live cell imaging demonstrated that both cell types we tested re-organize within the confluent cell sheet. Our MATLAB re-coloring tool, as has been seen previously\textsuperscript{15}, allows cells moving in the same direction to be more easily visualized (Figure I.3 – See Appendix I). Using our algorithm, we characterised the cell streams for one epithelial (ARPE-19) and one fibroblast (BJ) cell line, as shown in histograms in Figure 3.3A and B. We selected these cell types because we expected them to show differing migratory behaviour due to differences in the extent to which each cell type interacts with neighboring cells within the sheet: epithelial cells form stronger and more junctions between neighboring cells than fibroblasts. Surprisingly, both cell types showed similar behaviour: A large number of the cells do not participate in streams, as evidenced by the peak at $<20\mu m$ in Figure 3.3A and B, while for cells moving within streams, we observed a range of stream widths, with a peak at $40\mu m$, corresponding to a stream width of approximately two cells. Our analysis highlights the non-normal variation in stream widths for different cells within the sheet, suggesting a population average measurement is not necessarily an accurate representation of the behaviour of all cells within the sheet.

We also quantified the size of the streams using the standard velocity correlation function method (shown in Figure 3.3C and D). Figure 3.3C and D show data averaged for all cells and suggest that the radii at which cells become non-correlated (where the inverse cosine of the function levels off at approximately $\pi/2$) are approximately 200 and 250$\mu m$ for ARPE-19 and BJ cells, respectively. To allow easier comparison with our non-averaged data, we also quantified for each cell the radius at which the velocity correlation function approached 0 (specifically when it fell below 0.273 or $\theta = \pi/1.7$). This was selected to account for a non-perfect drop to exactly 0 in the function when cells become non-correlated. Figure 3.3E and F show the distribution of “stream widths” defined using this method. The distribution of stream widths calculated using the velocity correlation function method was wider than that found using our algorithm, and peaked at approximately 100$\mu m$ for both cell types as seen in Figure 3.3E and F, as opposed to the peak of stream width around 40$\mu m$ predicted by our algorithm as seen in Figure 3.3A and B. We attribute this difference to the impact of correlation along the stream length and the error this introduces depending on where a cell lies within the stream. The value returned by the velocity correlation function is a combined measure of both the length and width of the stream. Our algorithm, on the other hand, provides a novel method to quantify stream width specifically.
We also used our algorithm to quantify stream width in ARPE-19 cell sheets in the presence of a wound (supplementary Information figure 2 and movie SI2) and with cells expressing GFP-N-cadherin. Figure 5.3G and H show the stream width distribution for each situation. In the wound healing situation, the number of cells not participating in streams decreases and an increase in the number of cells participating in streams of 40 microns was observed. These observations are consistent with the initiation of directed collective migration on introduction of the wound. Peak stream width does not appear to significantly increase in contrast to what the track images suggest. This illusion is due to the directed motion of the cell in different streams following similar paths but at different time points. Interestingly, in cells expressing GFP-N-cadherin, where we expected increased cell-cell interactions, the distribution of stream widths shifted to lower values. This observation could be due to reduced cell motion in response to increased adhesiveness and further studies are currently underway to probe this effect and identify the key cellular parameters that determine stream width within a sheet of cells. Beyond the wounded and confluent sheets examples shown here we believe our algorithm will also be useful in the future for defining the impact on stream width of cells moving within confined spaces\textsuperscript{18}.

Our algorithm was written efficiently in C++ to allow researchers to run datasets of any size (varying number of timeframes) in minutes. The algorithm variables can be easily fine-tuned to quantify collective migration behaviour for any cell type. For example, it is easy to adjust parameters such as the number of frames to be analysed (based on how tortuous the cell migration path is) and the size of radii increments (based on average cell width). The program is fully automated, allowing analysis of the data for all of the wells from a 96-well plate with a single instruction, which is useful for integration with a high-content screening platform. On average, the program can run data containing approximately 2,400 cells (one site of a confluent well imaged at 4x magnification) over 24 time frames in approximately 20 seconds. Vast numbers of cells can therefore be analysed in a short time period, which is important as often computational time is an analysis bottleneck.
Figure 3.4 - Accuracy and sensitivity analysis of algorithm results: (A) Bar chart depicting the level of accuracy of the algorithm for measuring the stream widths of ARPE-19 and BJ cells. The accuracy of the wound healing assay and ARPE-19 cells expressing GFP-N-cadherin are also included. Error bars are 5% confidence intervals for n=30 cells. (B) Histogram of the percentage of ARPE-19 cells occupying streams of a given width for different ring thickness used in the algorithm. (C) Histogram of the percentage of ARPE-19 cells occupying streams of a given width for different angular definitions of correlations used in the algorithm. (B) and (C) demonstrate the sensitivity of the algorithm to these parameters.
We assessed the accuracy of our algorithm in estimating stream width by comparing its output to manual measures of stream width from our directionally colored images for randomly selected cells. Specifically, the stream widths of thirty randomly selected cells from three wells were evaluated manually for each cell type. Accuracy was computed as:

\[
Accuracy = \left[ 1 - \frac{\text{manual measurement} - \text{algorithm measurement}}{\text{algorithm measurement}} \right] \times 100 \quad [\text{Equation 2}]
\]

The average accuracy is displayed on the bar chart in Figure 5.4A and was not significantly different for each test case. The algorithm had a calculated accuracy of 89.7±8.9% for ARPE-19 cells, of 85.1±10.1% for BJ cells, 75.5±12.5% for ARPE-19 GFP-N-cadherin expressing cells and 82.2±10.5% for the ARPE-19 cells in the wound healing assay. The inaccuracy was a result of a combination of factors: Firstly, manually estimating the stream widths by hand was incredibly difficult due to the number of surrounding tracks, making our accuracy benchmark inherently inaccurate. This was a major contribution to the large deviation in accuracy, and further illustrates the need for an algorithm to evaluate stream widths. Secondly, errors may arise from cells that are not exactly in the centre or edge of a stream. In the algorithm used for all our experiments, to account for situations where the comparison cell being analysed is exactly in the center of a stream, the radius is doubled if both arcs finish at the same time; this was computationally easy to implement and was expected to improve the accuracy of the measurement. A similar calculation for cells located between the edge and centre of a stream would have unnecessarily increased the complexity of the code and hence was not implemented; therefore, these cells will report narrower stream widths. We do not expect this effect to be significantly detrimental to overall accuracy, however, unless assessing very wide streams. Still, this could be improved in future iterations of the algorithm. Thirdly, the algorithm may be too lenient when classifying neighboring cells as correlated. If a nearby cell moved in the same orientation for less than approximately 5µm, our observations determined the cell movement to be uncorrelated, whereas the program marked it as correlated, for that time point. This could be adjusted in the future by only considering data from pairs of cells that both move a minimum distance. Despite these factors, our algorithm reported stream widths that were much closer to those measured in experimental images than achieved using the standard velocity correlation function method.
We also performed a sensitivity analysis to determine the impact of the different algorithm variables on the results. Figure 5.4B shows a histogram comparing the stream width outputs for ARPE-19 cells if the radius increment is changed. The 10µm radius increment data shows similar peaking characteristics as the 20µm radius increment, but fails to detect larger stream widths, whereas the 40µm radius increment returns stream widths that are too high and inaccurate. The major differences arise due to under-sampling versus over-sampling: In smaller increments, the algorithm does not detect enough correlated cells to continue searching. In larger increments, the algorithm potentially assesses multiple streams within one arc. Increment size should be selected to correspond approximately to the width of one cell to get the most accurate results. We also assessed the impact of the angle used to define correlated movement (Figure 5.4C). We selected ±10° for correlation based on visual estimates of the angle between cells in a stream from our live imaging movies, but this could easily be adjusted to be more or less stringent. Decreasing this angle to 5° did not significantly change the shape of the stream width distribution or peak location, but did slightly increase the number of cells considered to be part of very narrow streams. Increasing this angle to 50°, however, significantly impacted the stream width distribution. Selection of an appropriate angle to define correlated movement, therefore, is also an important parameter for obtaining accurate results.

3.7 Acknowledgements

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3.8 References


Chapter 4
Non-Autonomous Contact Guidance Signalling During Collective Cell Migration

4.1 Commentary

The work described in this chapter was peer reviewed and published in the Proceedings of the National Academy of Science (PNAS) in 2014 (Londono, C. et al. Nonautonomous contact guidance signalling during collective cell migration. Proc Natl Acad Sci U S A 111, 1807-1812 (2014)). It was co-authored with M. Jimena Loureiro (co-first author), Benjamin Slater, Petra B. Lücker, John Soleas, Suthamathy Sathananthan, J. Stewart Aitchison, Alexandre Kabla, and Alison P. McGuigan (corresponding author). Author Contributions: CL, MJL, PBL and APM designed the study and wrote the manuscript with input from other authors. CL and MJL conducted all experiments and analysed the data with APM. BL provided MATLAB code for analysis and analysed data. JS performed the quantification of alignment. CL, SS, JSA and APM designed the interface grooves. SS did all photolithography and, with CL, measurements of grooves. AK created the computational model and ran all simulations.

Figure 4.1 – The migration behaviour of a cell within a confluent sheet is affected by the cues it receives from adjacent cells and from the underlying substrate: In this chapter, we explore how the migratory behaviour of epithelial and fibroblast cells is affected by interactions with their neighbours as well as topographic cues (grooves). Further, we examine the possibility that guidance from topographic cues can affect cells at a distance as a consequence of cell-interactions, and we find that the propagation of this topographic guidance cue is not dependent on tensional mechanisms and may be achieved solely through volume exclusion within a confluent cell sheet.

In this work, we provided evidence that guidance cues can be propagated in confluent sheets exclusively through mechanical cell-cell interactions that are not mediated by cell junctions or intracellular cell tension, both of which are commonly cited mechanisms for cell cooperation during collective cell migration. This suggested that volume exclusion mechanisms can be
sufficient to guide cell migration in dense populations, and supported an existing analogy of cells as a glass-like fluid.

4.2 Abstract

Directed migration of groups of cells is a critical aspect of tissue morphogenesis that ensures proper tissue organization and, consequently, function. Cells moving in groups, unlike single cells, must coordinate their migratory behavior to maintain tissue integrity. During directed migration, cells are guided by a combination of mechanical and chemical cues presented by neighboring cells and the surrounding extracellular matrix. One important class of signals that guide cell migration are topographic cues. While the contact guidance response of individual cells to topographic cues has been extensively characterised, little is known about the response of groups of cells to topographic cues, the impact of such cues on cell-cell coordination within groups, and the transmission of non-autonomous contact guidance information between neighboring cells. Here, we explore these phenomena by quantifying the migratory response of confluent monolayers of epithelial and fibroblast cells to contact guidance cues provided by grooved topography. We show that, in both sparse clusters and confluent sheets, individual cells are contact guided by grooves and show more coordinated behavior on grooved versus flat substrates. Furthermore, we demonstrate both in vitro and in silico that the guidance signal provided by a groove can propagate between neighboring cells in a confluent monolayer, and that the distance over which signal propagation occurs is not significantly influenced by the strength of cell-cell junctions, but is an emergent property, similar to cellular streaming, triggered by mechanical exclusion interactions within the collective system.

4.3 Significance statement

Group cell migration in response to guidance signals is a critical process in determining tissue organization. Here we explore the response of groups of cells to topographic guidance signals and reveal that guidance information can be transmitted between cells within the group. Significantly, we show that guidance information transmission is not dependent on cell-cell junctions or tensional forces within cells. Instead, we propose that signal transmission arises from a volume exclusion type mechanism and is an emergent property that can arise in dense cell populations.
4.4 Introduction

Directed collective cell migration is a fundamental process during embryo development\(^1\), adult organ regeneration\(^2\), wound healing\(^4\), and the progression of metastatic cancer\(^4\). During collective migration, individual cells must coordinate their motion both locally and globally, and move as a cohesive group with intact, often cadherin-based, junctions, and supracellular organization of the actin cytoskeletons of all cells within the group\(^4\). Mechanical cooperation of intercellular tensional forces via cell-cell junctions is thought to underlie coordination between neighboring cells during collective movement\(^5\)\(^-\)\(^7\). The local microenvironment of the cells provides the chemical and mechanical guidance signals that help direct collective migration. The rigidity and topographic texture of the local environment, as well as the tissue-level geometrical confinement of the cell population, can influence cell coordination and invasiveness\(^4\),\(^8\). Despite the fact that the topographic texture of the extracellular matrix is known to play a role in tumour cell dispersion into the surrounding tissue\(^9\), little is known about the role of topographic cues on the collective guidance of cell population \textit{in vivo} and \textit{in vitro}.

A number of cell types have shown contact guided migration along grooved substrates\(^{10\text{-}13}\) when cultured as single cells. The exact mechanism underlying contact guidance is not completely understood, but it is clear that, above a cell type-specific threshold groove depth, topographic features influence the organization of the actin cytoskeleton\(^{11,12,14\text{-}17}\) and of focal adhesions\(^{11,12,15\text{-}18}\), and that this altered organization is translated into directed migration\(^{11,13,19\text{-}22}\). In contrast to single cells, the collective response of cohorts of cells to topographic signals is not well understood\(^{12,23}\), in particular in confined space (defined here as no free space available within or surrounding the sheet) where cells are not experiencing a polarization cue from the presence of open space. Contact guidance within a group of cells is a more complex process than in the single cell case; in groups, changes in cytoskeletal organization induced by the topographic feature must be compatible with the cytoskeletal coordination required between neighboring cells. Furthermore, it is not known if each of the cells moving within a group responds autonomously to guidance cues, or if guidance signals can act non-autonomously (i.e. be transmitted between neighbors to act on cells with which they are not in direct contact) as a result of local steric interactions within the group and the need for local intercellular coordination.
Here, we set out to explore these fundamental questions by characterizing the guidance of individual cells and dense cell sheets in response to a topographic signal. We also presented confluent cell sheets with a hybrid surface containing a defined interface between a grooved and a flat region. This allowed us to determine if the contact guidance signal from the grooved region of the substrate would act non-autonomously through a sheet of connected cells to guide cell migration in non-grooved regions. Our results demonstrate that, within both clusters and confluent cell sheets, cells move in streams oriented along the grooves and that guidance signals can propagate within continuous cell sheets to neighboring cells that are not exposed to the topographic cue. Surprisingly, we found both experimentally and using computational modeling that the distance over which the guidance signal propagates is independent of junction strength, suggesting a non-tensional-based mechanism of cell-cell coordination underlies guidance signal propagation in confined monolayers. Furthermore, we show that signal propagation extends over a similar length scale as cooperative cell streams, and that both phenomena are dependent on similar physical parameters of the cells, consistent with a volume exclusion type mechanism.

4.5 Materials and Methods

*Grooved plate formation and characterization*

We assembled a 96-well plate with 48 flat bottom and 48 grooved bottom wells. The grooves were sinusoidal in shape, 1μm in pitch, and 152.7±1.5 nm (SEM) in depth (characterised by AFM, Fig. S1). Briefly, grooved features were created by replica molding polydimethylsiloxane (PDMS) (Sylgard 184 Silicone elastomer kit, Dow Corning Corporation, Midland, MI USA) at a 1:10 ratio of curing agent to base on a silanized holographic diffraction grating film (Edmund Optics, Barrington, NJ, USA). The cured PDMS sheet was then peeled off and used to generate a polyurethane mold from which PDMS replicas were created for assembly into 96-well plates (Figure 4.2A).

For some guidance propagation experiments assessing grooves parallel to the interface, we created plates in which half the well was flat and the other half of the well was grooved using the procedure outlined in Fig. S1B. A piece of diffraction grating film was taped onto a glass slide and spin coated with MICROPPOSIT® S1811® positive resist (Shipley, Malborough, MA, USA) for 30 s at 6100 rpm resulting in a ~1.7 μm thin layer of photoresist on the film. The photoresist was soft-baked on a hotplate at 100 °C for 10 min. Subsequently, the film was patterned using
UV light with an exposure energy of 155 mJ/cm² through a photomask with alternating 9 mm wide black and blank lines. The photoresist was developed by immersion in MF321 developer (Shipley) for 20 min. Parts that were exposed to UV light washed off, whereas parts that were not exposed stayed on the film covering the grooves and thus creating a flat area. The patterned film was then rinsed with de-ionized water and dried with nitrogen. The height of the step associated with the interface was quantified using a profilometer (Tencor Alphastep 200, KLA-Tencor, CA, USA). PDMS molds were then created from this master as described above. This method led to the presence of a 1 µm groove at the interface between grooved and flat regions which was not suitable to test the perpendicular propagation case.

To create interfaces without the 1 µm step at the interface, we used the following procedure. A thoroughly cleaned and dried Si-wafer was spun-coated first with adhesive primer hexamethyldisilazane (MicroPrime MP- P20 HMDS, Shin-etsu, Tokyo, Japan) and then with S1811 positive resist at 4000 rpm for 40 s. The photoresist was soft-baked at 105 °C for 2 min. Photolithography was done on the resist-coated wafer using a chrome-glass photomask with regions containing 2 µm- pitch lines. The photoresist was exposed to UV light (365 nm wavelength, 16.9 mW/cm² intensity) in contact mode for 10 s, using an MA6 Mask Aligner (SÜSS MicroTec AG, Sunnyvale, CA, USA). The unexposed photoresist was removed by MF-321 developer. Dry etching was carried out using inductively coupled plasma reactive ion etcher (Phantom II RIE/ICP System, Trion Technology, Tempe, AZ, USA), at a pressure of 100 mTorr and RIE RF power of 120 W with SF6, O2, CHF3 and He etchants at 30 sccm, 20 sccm, 12 sccm and 10 sccm flow rates respectively, and these settings resulted in an overall etch rate of 400 nm/min. Etching times were adjusted to alter final groove depth and spacing. Prior to soft-lithography, patterned Si wafers (~630nm or ~3.14µm in depth, characterised by AFM) were washed with acetone and then silanized in a desiccator. Replica molding to obtain grooved substrates was achieved as described above.

Cell culture and live cell imaging

We conducted experiments using human retinal epithelial ARPE-19 cells (ATTC, Manassas, VA, USA) and human foreskin fibroblast BJ cells (ATTC). ARPE-19 were grown in Dulbecco’s modified Eagle’s medium/nutrient F-12 (DMEM/F-12) (Invitrogen, Burlington, Ontario, Canada) supplemented with 10 % fetal bovine serum (FBS) (VWR, Canada) and 1 %
Penicillin/Streptomycin (VWR). BJ were grown in DMEM (ATCC) supplemented with 10% FBS and 1% Pen/Strep. All cells were maintained in a humidified atmosphere at 37 °C and 5% CO2. Prior to seeding, PDMS culture wells were coated with a 10 µg/mL solution of stabilized fibronectin (Biomedical Technologies, Inc., Stoughton, MA, USA) for 1 h, followed by a PBS rinse. Cells were then seeded at the indicated densities and allowed to attach for 24 hrs before conducting tracking experiments.

For experiments on wells that were all flat or all grooved, we seeded 22,000 or 65,000 cells/cm² in a 96-well plate. Wells in rows A and H and columns 1 and 12 of the plate were filled with 200 µL of PBS and were not used for cell experiments to avoid edge effects arising from temperature or humidity differentials. For experiments using hybrid surfaces containing an interface between flat and grooved regions, we seeded 86,000 cells/cm² (for low density experiments) or 156,000 cells/cm² (for all other conditions). These increased seeding densities were necessary to ensure that we generated a confluent sheet with continuous junctions across the interface.

Prior to live cell imaging, cells were stained with 500 ng/mL Hoechst 33342 (Invitrogen, Burlington, Canada) in cell culture medium for 30 min. An ImageXpress Micro high-content screening microscope with a live-cell imaging module (Molecular Devices, Sunnyvale, USA) was used to image the cells every 20 min for 8 h.

Cell migration and signal propagation analysis

Cell tracking was performed using the Multi-Dimensional Motion Analysis application module in the MetaXpress software package (Molecular Devices, USA). This tracking algorithm uses nuclear shape and fluorescent intensity measurements to track the position of individual cells over time, from which cell speed (total travel distance/time), cell velocity (net displacement/time), persistence (velocity/displacement) and angle of migration of each cell could be determined. Median values of speed or velocity were obtained for each well due to the non-normal distribution of the data. The mean of all the well medians from each test condition was then calculated. Coordination of migration between neighboring cells was quantified using a custom-built algorithm (described elsewhere in detail (1)). Specifically, we calculated the distance over which each cell showed coordinated movement (movement in directions ±10 °) with neighboring cells. Based on cell size, stream widths of distances of 40 µm or more represent
groups of more than 2 cells. We quantified, for grooved and non-grooved substrates, the percent of total cells with distances of coordinated movement of 40 μm or greater to obtain the percentage of cells participating in streams. We calculated the average stream width by taking the average stream width of all cells in streams greater than 40 μm in width.

For every well in every experiment, spatial correlation numbers were calculated as a function of distance from each cell and were averaged across all cells across all time intervals (via Equation 3 below). The angled brackets signify an average across all cell pairs and time.

\[
V(r) = \langle \frac{\vec{v}_i \cdot \vec{v}_j}{|\vec{v}_i||\vec{v}_j|} \rangle, \quad [\text{Equation 3}]
\]

In each time interval, the mean velocity calculated from all of the cells was subtracted from each individual cell velocity before calculating the correlation numbers. These correlation numbers were then fit as an exponential and the correlation length was taken to be the inverse of the decay constant.

**Guidance signal propagation quantification method**

To quantify the propagation distance in the interface experiments, an algorithm was written in MATLAB to evaluate the alignment of the velocity vectors (calculated at five 1.5 hour intervals for each cell) of the moving cells with the groove direction as a function of distance from the interface. In 40 μm sized bins, the average velocity of the moving cells in each time interval was calculated for bins up to and including 720 μm from the interface in both the grooved and flat regions. An average velocity vector calculated across all of the time intervals for each bin was then evaluated. By calculating the sine of the velocity angle (cosine in the perpendicular interface experiments), the extent at which the migration of the cells in each bin is in alignment with the groove direction could be determined. Because cells on grooves are constrained to move along the grooves but with no preferred direction, their average velocity cancels out. To avoid this, we squared the sine or cosine values. Because the average value of sine or cosine squared in completely random migration is 0.5 (i.e. \(\sin^2(45°)\)), we subtracted this value from all calculations to define the value of randomly migrating cells as 0. All of the alignment numbers were then normalized by dividing them by the average alignment of the cells moving on the grooves. By this definition, a value of 1 implies perfect alignment (cells are moving 100% in the groove...
direction) and 0 implies that cell movement is completely random. The propagation distance was recorded as the binned distance just before the alignment number first became \( \leq 0.321 \) (value for \( \sin^2\theta - 0.5 \) at the point where the average cell velocity deviates more than 25°).

**Junction and tension modifications**

Adherens junctions were disrupted by antibody inhibition using modifications to a previously published protocol\(^{34}\). Specifically, cells were serum-starved for 30 min, after which they were incubated in 4 mM EGTA in serum-free medium. After a PBS wash, 3 \( \mu \text{g/mL} \) E-cadherin antibody (clone SHE78-7) (Millipore, Billerica, MA, USA) in serum medium was added to the cells. For control experiments, 3 \( \mu \text{g/mL} \) FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA) were added instead. Following 30 min incubation, cell migration experiments were conducted. GFP-N-Cadherin over-expressing cells were produced by transduction of ARPE-19 with a lentivirus containing a GFP-N-cadherin plasmid. Cells were then FACS sorted for GFP. Levels of N-Cadherin staining at the cell membrane were quantified using ImageJ (3). For each image, the fluorescence intensity was measured along the membrane of 5 cells in 20 spots per cell with the spot size equaling approximately the width of the membrane.

Myosin II activity was decreased with 5 \( \mu \text{M} \) of Blebbistatin (Sigma-Aldrich), a myosin II ATPase inhibitor, which remained in solution during cell tracking. In order to increase myosin II activity, cells were treated with 0.25 nM of Calyculin A (Cell Signalling Technology, Beverly, MA, USA) for 30 min prior to cell tracking experiments. Calyculin A is a type II phosphatase inhibitor that is specific to myosin light-chain phosphatase at low concentrations, thus promoting activation of myosin II.

**Immunocytochemistry**

Cells were fixed with 4 % paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min, and rinsed three times with PBS. The cells were then permeabilized using 0.1 % Triton-X 100 (Sigma-Aldrich) at room temperature for 20 min. Following permeabilization, cells were stained with rhodamine phalloidin (Invitrogen Life Technologies, Burlington, Ontario, Canada) for 2 h at room temperature to assess F-actin. To determine microtubule structure, a mouse monoclonal antibody to the \( \beta \)-subunit of tubulin (Abcam, Cambridge, MA, USA), was added at a dilution of 1:1000 overnight at 4 °C. To image cellular tight junctions in wild-type
ARPE-19, a mouse monoclonal antibody to zona occludens-1 (ZO-1) (Invitrogen) was used at a dilution of 1:1000 overnight. To image N-Cadherin, cells were incubated with mouse monoclonal (clone 2G7) N-Cadherin antibody (Abcam) at a dilution of 1:100 overnight at 4 °C. After washing three times with PBS for 5 min, all mouse primary antibodies were conjugated with an Alexa 488 anti-mouse secondary antibody (Invitrogen) at a dilution of 1:250 for 45 min at room temperature. To image cellular junctions in mouse antibody-treated cells, cells were fixed in methanol at -20 °C for 15 min, and rinsed with PBS three times. Rabbit anti-ZO-1 (Invitrogen) was then applied at room temperature for 3 h, followed by rinsing with PBS three times. An Alexa 488-conjugated secondary anti-rabbit antibody was applied at 1:160 dilution for 2 h at room temperature. Prepared samples were imaged on an Olympus IX-81 inverted laser scanning confocal microscope using a 40x/0.8 LUMPlanFI dipping water objective or an Olympus IX-81 inverted microscope using 32x or 64x magnification.

Modeling cell streaming and signal guidance propagation

A numerical model of self-propelled cells was used to study cell interactions across the boundary between grooved and flat domains. The algorithm used in this study is based on a Cellular Potts Model and has been characterised in detail in29. Cells mechanically interact through an interfacial energy term (encompassing the cell's shear modulus and adhesion) and excluded volume interactions. Cell migration is caused by a propulsive force of constant magnitude on the substrate. The direction of the motile force evolves as a result of a feedback from the cell displacement to the polarity of the cell; such feedback involves a characteristic persistence time. The relative values of the interfacial energy, the motile force and the persistence time of the cell polarity directly control the emergence of collective behavior. The latter has been quantified in the model in terms of the correlation length of the cell displacements in flat conditions, i.e. the typical distance between cells below which their directions of migration are correlated (an alternative measure of stream width). To calibrate the model with the experimental data, the correlation function of the velocity field in the experimental system was measured (Figure 4.14). We found a correlation length of the order of 7 cell diameters was required in the model to best match the experiment. The following values, as defined in29, produced such a correlation length: surface energy J=5, motile force μ=0.175, noise T=2.5 and persistence time τ=10 Monte-Carlo time steps.
For our simulations, cells were enclosed in a domain of 74x64 cell diameters in size. Cells located on a strip of width 10 cell diameters on the left side of the domain were biased to move along $y$, mimicking locally the effects of the grooves on the cells. The grooved domain was periodic in the $y$ direction, i.e. along the interface direction. To test the influence of the correlation length (a measure of stream width) on the signal propagation distance, confluent layers were generated and the value of the motile force was varied keeping all the other parameters constant. To create either cohesive or non-cohesive populations, the surface energy of unbounded cell membrane was set to 5 or 2 respectively. The density of the cell population was controlled in the model by creating homogeneous gaps in the initial cell sheet configuration. To focus on the steady state dynamics, simulations were run for up to 20,000 Monte Carlo Time Steps and flow profiles were analysed between 3,000 and 20,000 time-steps and then averaged over time. The propagation distance was measured as the distance between the interface and the position where the mean vertical velocity component (parallel to the interface) falls below 0.5.

Statistics

To test for significant differences between two test groups, we first used an F-test to determine if equal variance could be assumed and then a pooled or non-pooled Student's t-test to identify significant differences between test groups. To test for the significance of correlation coefficient values, calculated R values from linear regression analysis were compared to critical R values for n-1 degrees of freedom from a table of critical Pearson coefficients. For all tests, p-values of less than 0.05 were considered significant.
4.6 Results

4.6.1 Guidance of cell migration by grooves

Figure 4.2 - 96-well grooved plate fabrication and characterization: A) Schematic of method to create completely grooved and flat wells with the plate containing 48 wells of each type. B) Schematic of fabrication approach for a 96-well plate containing wells that are half grooved and half flat. Color gradient indicates grooved regions C-D) Scanning electron micrographs of the grooved-flat interface with the grooves (C) parallel and (D) perpendicular to the interface. Scale bars are 10 μm. E) AFM surface profile of PDMS substrates, showing the interface between flat (left) and grooved (right) sections.
Figure 4.3 - Quantification of cell migration and coordination behavior on flat versus grooved substrates: Cell migration characteristics of ARPE-19 and BJ cells at sparse and confluent cell densities on flat versus grooved substrates. (A-B) Distribution of cell migration direction on flat versus grooved substrates for (A) ARPE-19 and (B) BJ cells. The peak created by the grooved substrates between -25° and 25° indicates cell migration guidance. (C) Cell speed was significantly increased by grooves in sparse ARPE-19 and BJ cells, but not in confluent cell sheets of ARPE-19 or BJ cells. (D) Cell velocity was significantly increased by grooves in sparse ARPE-19 and BJ cells and in confluent cell sheets of ARPE-19 and BJ. (E) Cell persistence was significantly increased by grooves in sparse ARPE-19 and in confluent cell sheets of ARPE-19 and BJ but not significantly different in sparse BJ cells. (F) Grooves significantly increased the percentage of cells participating in streams of width greater than 40 µm for both ARPE-19 and BJ cells. (G) Grooves significantly increased stream width in both ARPE-19 and BJ cells. (H) Grooves significantly increased correlation length in ARPE-19 but not BJ cells. Error bars represent 95% confidence intervals.

We tracked the migration paths of individual ARPE-19 (Human retinal pigment epithelial cells) and BJ (Human foreskin fibroblast) cells at both sparse and confluent densities on flat and grooved topography using a custom-built grooved 96-well plate (Figure 4.2A). ARPE-19 cells were selected because they form confluent epithelial monolayer sheets, while BJ cells were selected to assess the behavior of a non-epithelial cell type in which continuous cell-cell junctions are not formed within the sheet24. On grooves, both ARPE-19 and BJ cells migrated parallel to the direction of the grooves (Supplemental Movie S4.1), as demonstrated by the large peaks between -25 and 25° in the distribution of cell migration angles shown in Figure 4.3A and B. As a measure of guidance effectiveness, we quantified the percent of cells within this peak (i.e. within the range -25 to 25°). At sparse densities, 52.1% of ARPE-19 and 70.7% of BJ cells migrated in a direction parallel to the grooves. This effect was more pronounced at confluent densities, with 59.1% of ARPE-19 and 80.6% of BJ cells lying within the peak. This data suggests that grooves are capable of producing robust guidance of cell migration at both sparse and confluent cell densities in the epithelial and fibroblast cell types tested.
4.6.2 Grooved topography increases confluent cell velocity and persistence but not speed

![Alignment of actin in response to grooves](image)

**Figure 4.4 - Alignment of actin in response to grooves:** A-B) ARPE19 cells seeded sparsely on grooved and flat PDMS show A) unaligned F-actin on a flat substrate, and B) aligned F-actin in the direction of the grooves (white arrow) on a grooved substrate. C-D) ARPE19 cells seeded at confluent densities also show C) unaligned F-actin cytoskeleton on flat PDMS, but D) aligned F-actin on grooves. All scale bars are 40 µm.

We next sought to quantify the impact of contact guidance on the migratory properties of cells. We quantified cellular speed (total distance travelled over time), velocity (net distance travelled over time) and persistence (velocity divided by speed) of cells on flat versus grooved substrates. As observed previously\(^{22,25}\) at sparse densities, cell migration speeds were moderately enhanced on grooves compared to flat substrates by 10.5 and 14.4 % for ARPE-19 and BJ cells, respectively. No significant differences were observed, however, for confluent cells (Figure 4.3C). This occurred despite the fact that, at both seeding densities, we observed alignment of the actin cytoskeleton with the grooves (Figure 4.4), which has been hypothesized to account for more efficient migration, and hence increased speed, in sparse cells migrating on grooves\(^{22}\). In contrast, cell velocity was significantly increased on grooves at both cell densities (Figure 4.3D). Consistent with this increase in velocity, the grooved substrates increased persistence in confluent cells by 16.5 % and 14.5 % for ARPE-19 and BJ cells, respectively (Figure 4.3E), indicating that within confluent sheets, grooves cause individual cells to move in a more directed fashion.
4.6.3 Grooved topography increases intercellular coordination

We next wanted to determine if contact guidance impacted cell-cell cooperation between neighbors within confluent sheets. Groups of epithelial cells within confluent sheets have previously been described to form cooperative “streams” whose internal dynamics are reminiscent of glass-like behavior\textsuperscript{26,27}. Narrow streams of coordinated groups of cells formed within confluent sheets of both ARPE-19 epithelial cells and BJ fibroblast cells on both flat and grooved substrates (Figure 4.5). As a quantitative measure of cell-cell cooperation, we quantified both the percentage of cells migrating in streams (i.e. cells in streams ≥ 40 µm or more than two
cells wide) and the width of the streams. We also calculated a correlation length, which assesses the distance over which cell behavior is coordinated\textsuperscript{28}. Grooves significantly increased the percentage of cells participating in streams (Figure 4.3F), stream width (Figure 4.3G) and the correlation length (in ARPE-19 cells only) (Figure 4.3H). However, the increase in the number of cells that participate in streams and the distance over which streams extend could simply be attributed to the response of individual cells. By restricting the motion of the cells to directions parallel to the grooves, the number of cells moving in the same direction could increase independently of any increased coordination between neighboring cells. It is therefore unclear from this result whether the increased cooperation observed arises from autonomous but restricted directed motion of individual cells within the sheet, or if the guidance signal from the grooves acting on one cell propagates to non-autonomously guide neighboring cells and increase cell-cell coordination.
4.6.4 Propagation of contact guidance signals from a topographic feature

Figure 4.6 - Quantification of guidance propagation from a topographic feature: Guidance propagates about nine cell layers. Groove depth, adherens junctions and myosin activity do not impact propagation distance. (A-B) Migration tracks of cells located on the flat region at start of the experiment on surfaces with (A) grooves perpendicular or (B) grooves parallel to the interface. Tracks are colored according to their angles (guided (red) versus non-guided (blue)). (C) Quantification of alignment of cell migration direction with groove direction as a function of distance from the interface for grooves parallel or perpendicular to the interface. (D) Propagation distance from the interface, quantified as the distance at which the curves in (C) reach 0.321 (i.e. cells no longer move within 25° of the groove direction) for grooves parallel and perpendicular to the interface. (E) Propagation of guidance signals from the interface for parallel grooves in GFP-N-Cadherin over-expressing ARPE-19 cells, anti-E-Cadherin-treated ARPE-19 cells, Blebbistain (BB) treated cells and Calyculin A (CalA) treated cells. No significant difference in signal propagation distance was observed between treated and control cells. Measurements were made on at least 19 wells from at least 3 independent experiments. (F) Propagation distance from the interface measured for parallel grooves of different depths and for cell sheets at different densities. No significant difference in signal propagation distance was observed between deep and shallow grooves. Sheets with a high cell density did however show a significantly higher propagation distance than cells in low density sheets.
To better understand the impact of topographic cues on cell-cell coordination and distinguish between autonomous and non-autonomous guidance effects, we next set out to determine if guidance signals can propagate from a groove feature through a sheet of cells to induce guided motion of cells some distance from the groove feature. To do this, we created hybrid substrates with a defined interface between flat and grooved regions (Figure 4.2B). We cultured cells as a continuous confluent sheet over the whole substrate, and quantified cell migration at the interface region between the grooved and non-grooved sections of the substrate. To investigate guidance propagation, we determined how far from this interface cells migrated in a guided manner.

**Figure 4.7 - Assessment of cell sheet formed over grooved-flat interface.** A-C) Visualization of cell sheet at grooved-flat interface, which is indicated by the arrowheads at top and bottom edges of the images. The direction of the grooves is indicated by the double-headed arrows. A) DIC image reveals location of grooved-flat interface, in the centre. Cells form a continuous monolayer across the interface as seen through B) the formation of tight junctions, and C) the continuity of the actin cytoskeleton. D) Quantification of percentage of cells with F-actin aligned in a direction ±250 of the grooves at different cell distances from the interface. Randomly oriented cells should theoretically show ~27.7% of cells with alignment in the direction of the grooves. Three cells away from the interface (where 22% of cells show aligned actin) the orientation of the actin can therefore be considered random. Quantification was done from 10 images with at least 41 cells analysed at each distance from the interface. All scale bars are 40 µm.
Continuous ARPE-19 cell sheets showed normal morphology (Figure 4.7A-C) within the cell sheet on the hybrid surfaces, and cell-cell junctions were not disrupted over the interface. Cells on the grooved side of the interface and on the interface itself showed aligned actin, while cells on the flat side, as little as three cells away from the interface, showed isotropically distributed actin (quantification is shown in Figure 4.7D). We tracked cell migration at grooved-flat interfaces for substrates with grooves parallel or perpendicular to the interface and colored cell tracks red if migrating within ±25° of the groove direction—or blue otherwise (Figure 4.6A and B). In both cases, cells on grooves showed guided behavior, while cells on flat showed an isotropic distribution of movement direction. The transition from guided to random cell migration however, was not exactly at the flat-grooved interface. To determine where the transition from guided to random cell migration occurred, we quantified the component of the velocity vector parallel to the groove direction as a function of the horizontal position within the sheet (Figure 4.6C) and scaled values between 0 (fully random movement) and 1 (aligned migration). The distance at which this scaled value dropped to 0.321 (i.e. when the average cell velocity vector deviated more than 25° from the groove direction) was defined as the propagation distance (see Section 4.5 - Materials and Methods for calculation details).

When grooves were oriented perpendicular to the interface, groups of cells from the flat region randomly migrated over the interface onto grooved regions and became guided by the grooves (Figure 4.6A). This transition from random to guided migration as cells cross the interface led to increased migration of cells on flat substrate regions towards the interface extending several rows of cells (Figure 4.6C and D). The average distance from the interface at which cells continued to migrate in a directed fashion was 217 ± 29 μm (95% confidence intervals) or 9 ± 1 cell lengths (cells are elongated perpendicular to the interface) into the flat substrate. We speculate that, as groups of cells randomly cross the interface, they migrate along the grooves in a directed fashion, pushing or pulling cells located at the vicinity of the interface; this biases the migration of the cells proximal to the interface, leading to funneling of the cells across the flat-groove interface and disruption of random migration in a portion of the flat region. When grooves were oriented parallel to the interface, however, cells tended not to cross the interface between the grooved and flat region (Figure 4.6B). Guided cell behavior was still observed to propagate a distance of 111 ± 12 μm (95% confidence interval) or 9 ± 1 cell widths (cells are elongated parallel to the interface) beyond the grooved-flat interface into the flat region of the
substrate, suggesting that cells do not need to cross the interface and make contact with the groove for aligned cell migration to occur on the flat region proximal to the interface (Supplemental Movie S4.2). In this case, guidance arose only from propagation of the guidance signal between cells in contact with the grooves and cells located on flat regions of the substrate (Figure 4.6D), allowing us to better probe the mechanism of this non-autonomous guidance.

Figure 4.8 - Characterization of cells expressing GFP-N-Cadherin. (A-D) Confocal images of N-Cadherin (green), nuclei (blue) and F-actin (red) in (A, C) wild type and (B, D) N-cadherin over-expressing cells. (E) Quantification of fluorescence levels at cell-cell junctions in wild-type and GFP-N-Cadherin over-expressing cells. Significantly higher levels of N-Cadherin were present at the cell membranes of GFP-N-Cadherin expressing cells (*p=7.96 x 10-5). (F) Impact of N-Cadherin over-expression on cell speed. N-Cadherin over-expressing cells (NCad) migrated at significantly slower speeds than wild-type cells (*p=8.6 x 10-10), as expected due to increased adhesion between the cells. Error bars represent 95% confidence intervals. Arrows indicate the direction and location of grooves in the images. Scale bars are 40 µm for (A-B) and 20 µm for (C-D).
Figure 4.9 - Characterization of anti-E-Cadherin treated cells. (A-B) Phase contrast images showing changes in junction contrast on treatment with (A) non-specific anti-FLAG and (B) anti-E-Cadherin. Spaces between the cells in (B) are more distinct, suggesting junction disruption with the anti-E-cadherin treatment. (C-D) Actin distribution in cells treated with (C) non-specific anti-FLAG or (D) anti-E-Cadherin. Peripheral actin is less concentrated in anti-E-Cadherin treated cells consistent with junction disruption. (E-H) ZO-1 distribution in cells treated with (E, G) non-specific anti-FLAG or (F, H) anti-E-Cadherin. ZO-1 (green), a marker of tight-junction formation, is disrupted in anti-E-Cadherin treated cells consistent with junction disruption. (I) Impact of junction strength reduction on cell speed. No significant difference in cell speeds between treated and un-treated cells was observed (p = 0.717). Double-headed arrows indicate the direction of grooves while arrowheads mark the location of the interface in the images. Scale bars are 25 µm for (A-F) and 40 µm for (G-H).
Based on existing models that describe tension-based mechanisms of cell-cell coordination\(^5\)\(^-\)\(^7\), we hypothesized that tensional forces transmitted between the cells via cell-cell junctions would underlie this guidance signal propagation and that the distance from the interface that the guidance signal propagates should depend on junction strength. To test this hypothesis, we characterised signal propagation in ARPE-19 cells with modified adhesion and contractility. Specifically, we over-expressed GFP-N-Cadherin to increase cell-cell adhesion (over-expression of N-Cadherin levels at membrane and impact on cell migration properties is quantified in Figure 4.8), and disrupted junctions by incubation in medium with a calcium chelator followed by incubation with anti-E-Cadherin antibodies to prevent re-formation of the junctions (junction marker expression is characterised in Figure 4.9). Surprisingly, we found that neither increasing nor decreasing cell-cell adhesion impacted the signal propagation distance within the sheet (Fig. 6.2E). To further explore the influence of tension, we measured propagation distance in cells treated with Calyculin A or Blebbistatin to increase or decrease myosin activity (Figure 4.10), and the ability of the cells to generate tension, respectively. Consistent with our observations of cells with modified junction strength, neither treatment significantly changed the distance over which the guidance signal propagated (Figure 4.6E), providing evidence that a non-tensional based mechanism dominates signal propagation in this context.

We next asked if geometric parameters in the system could influence signal propagation. Since groove depth has been shown to be the key geometric feature that influences contact guidance of migration in single cells\(^13\), we first assessed if groove depth impacted propagation distance but found that propagation measurements made on deep versus shallow grooves showed no
significant differences (Figure 4.6F). We next hypothesized that signal propagation could result from tight packing of the cells in the sheet since space availability has previously been shown to impact cell migration and coordination behavior. We therefore conducted experiments at high and low cell densities to be able to vary cell size and change the sheet packing. We found that decreasing cell density (and therefore increasing cell area) significantly decreased the distance of signal propagation (Figure 4.6F). This suggested that sheet architecture and limited free space available for cell re-organization could provide a non-tensional based mechanism for signal propagation.

### 4.6.5 Cellular streaming model of signal propagation

![Figure 4.11](image)

**Figure 4.11 - Propagation distance predictions from computational modeling:** (A) Cell velocity field from computational model. Cells to the left of the interface (indicated by the dashed line) were biased to move vertically, mimicking cells on grooves. No bias was applied to cells to the right of the interface. Arrows are placed at the centre of each cell within the sheet. Colors indicate the direction of migration. (B) Mean vertical velocity as a function of the distance from the interface for parallel grooves case. The data is normalized so that the mean vertical velocity on the grooves is 1. The propagation distance is defined by the intersection between the curve and 0.321. (C) Propagation as a function of cell density for wild type (blue) and cadherin-inhibited (red) cells. (D) Propagation distance as a function of the correlation of the cell population. According to the model, a correlation exists between propagation distance and correlation length. Error bars represent 95% confidence intervals.

To further probe the possibility that the underlying mechanism of signal propagation originates from steric constraints due to limited free space, we adapted an existing computational model of cell streaming in confluent sheets to model cell behavior on our hybrid substrates in the
parallel case configuration. The main biophysical parameters in the model are (i) membrane energy of cell-cell junctions and any unbound membrane (controlling the cohesion of the cell population), and (ii) the motile force that cells exert on the substrate and persistence of cell polarization. The volume of each cell is constrained by a high incompressibility, enabling steric mechanical interactions between neighboring cells due to volume exclusion. Two different domains were introduced: cells on the grooved region were modeled by biasing their migration direction to be parallel to the interface, whereas cells in the flat region had no preferred velocity direction. Model parameters for each cell type (wild-type and cells with modified junction strengths) were derived from our experimental measurements of bulk migration dynamics on flat substrates. We then analysed how far guided migration propagated from the grooved domain into the flat domain. Consistent with experimental observations, our model predicted guided migration extending past the grooves, decaying to random behavior ten cell diameters into the flat region (Figure 4.11A and B, Supplemental Movie S4.3) in the parallel interface case. Furthermore, for cells with reduced junction strength (and hence cohesion), we found that as long as the cell density remained above 80% of confluence, there was no difference in guidance signal propagation distance compared to wild-type cells and that steric mechanical interactions alone, arising from the incompressibility of the cells, were sufficient to propagate the directional effect of the grooves (i.e. movement within ±25° of the grooves) up to 6 cells away from the boundary (see Figure 4.11C and Supplemental Movie S4.4). Although the representation of cohesive forces is very simplistic in the model, this observation nevertheless shows that steric mechanical interactions due to cell incompressibility are sufficient to trigger collective effects and propagate the guidance signal away from the interface and that, consistent with our experimental observations, this effect is independent of junction strength.

4.7 Discussion

Previous work has extensively characterised contact guidance in single cells. Here, we describe the collective response of confined confluent cell sheets to grooved topographic guidance signals. We found that interactions between neighboring cells do not prevent guidance by the grooves and that individual cells within the confluent sheet migrate in narrow streams ~50 μm wide (three or four cells), parallel to the direction of the grooves. As with single cells, the presence of the groove guidance signal increases velocity and persistence in confluent cell sheets. Unlike in sparse cell experiments, however, grooves do not increase migration speed in
confluent cell sheets. Increases in sparse cell migration speed on grooves have previously been explained by the increased organization of the cytoskeleton leading to more efficient migration\textsuperscript{22}. However, on grooved substrates, not only sparse but also confluent cells exhibited actin alignment parallel to the grooves. This suggests that either, changes in cytoskeletal organization induced by the grooves in confluent cell sheets are not sufficient to increase cell speed, or that factors alternative to actin organization, such as cell junction properties or lack of free space, limit maximum cell speed in confluent sheets. Of these two possibilities, lack of free space seems most likely, given that that reducing cell junction strength has no effect on cell speed within confluent sheets (Figure 4.9).

In addition to increases in cellular persistence and velocity, grooves increased the percentage of cells moving as streams, the average stream width and the average correlation length (for ARPE-19 cells only), all measures of cell-cell coordination within confluent sheets. Furthermore, we found that topographic cues have the potential to impact cell-cell coordination beyond just autonomously limiting the migration direction of individual cells and that the impact of this propagation depends on groove orientation. When monolayers were observed on hybrid surfaces containing a sharp interface between grooved and non-grooved regions in which the grooves were perpendicular to the interface, cells from flat regions that randomly crossed the interface induced the movement of neighboring cells to also cross the interface and become guided by the grooves. When the grooves were parallel to the interface, cells moved along the interface, rarely crossing it, and guidance information from grooved regions was transmitted between neighboring cells to impact cells on flat regions that never make contact with the groove. Interestingly, in this situation, guidance of cells in flat regions proximal to the grooved-flat interface appears to be an emergent property characteristic of groups of densely packed cells, and is not due to alignment of the actin cytoskeleton, since cells more than three cells from the interface did not show aligned actin (Figure 4.7D). Furthermore, increasing groove depth, which is known to increase extent of cell alignment with the groove, and likely therefore actin alignment\textsuperscript{31}, had no impact on propagation distance. Surprisingly, the distance over which signal propagation occurred was also independent of cell junction properties or tension within the cell (Figure 4.6F and Figure 4.11C).
Figure 4.12 - Correlation lengths in anti-E-Cadherin-treated ARPE-19 cells and GFP-N-Cadherin over-expressing ARPE-19 cells, Blebbistain (BB) treated cells and Calyculin A (CalA) treated cells. All measurements were made on cells on flat substrate regions. No significant difference in correlation lengths between treated and control cells was observed (p=0.58 for GFP-N-Cadherin, p = 0.71 for anti-E-Cadherin-treated cells, p = 0.08 for BB treated cells and p = 0.93 for CalA treated cells). Measurements were made on at least 17 wells from at least 3 independent experiments.

To probe alternative non-tension based mechanisms that may explain the observed guidance signal propagation behavior, we compared the properties of signal propagation to cellular streaming, a cooperative process observed in dense cell sheets. Cell streaming also requires cell-cell coordination and is observed above a threshold cell density\textsuperscript{32}, and has previously been compared to behavior in crowded particulate systems\textsuperscript{26,33}. In such systems, cooperative movement arises due to volume exclusion and forced re-organization of the particles within a confined space. Consistent with this non-tension based description of streaming, we found that modifications to junction strength and myosin activity (tension) have no significant effect on streaming correlation length (Figure 4.12). By characterizing the behavior of non-cohesive cell populations \textit{in silico}, we also observed that simple steric interactions are sufficient to trigger collective effects, spatial correlations, and signal propagation (Figure 4.11C). We therefore propose that guidance signal propagation in confined confluent cell sheets arises from the same volume exclusion phenomenon that produces cell streaming.
Figure 4.13 - Identifying relationships between propagation distance, correlation length, cell density and cell speed from in vitro data: (A) Correlation length as a function of average cell area measured from cells on flat substrate regions showing a significant correlation ($R=0.538$ versus $R_{\text{significant}}=0.195$ for $n=127$). (B) Signal propagation distance as a function of average cell area measured at the interface showing a significant correlation ($R=0.241$ versus $R_{\text{significant}}=0.138$ for $n=154$). Propagation distance being measured in 40µm bins results in the observed banding/segregation of the data. (C) Correlation length as a function of median cell speed measured from cells on flat substrate regions showing a significant correlation ($R=0.623$ versus $R_{\text{significant}}=0.195$ for $n=127$). (D) Signal propagation distance as a function of median cell speed (speed measurements made on flat substrate regions) showing a significant correlation ($R=0.284$ versus $R_{\text{significant}}=0.195$ for $n=116$). (E) Propagation distance as a function of correlation length (correlation length measured from cells on flat substrate regions) showing a significant correlation ($R=0.214$ versus $R_{\text{significant}}=0.138$ for $n=153$).
Figure 4.14 - Assessment of cell speed impact using numerical model. (A) Relationship between the propagation distance and the cell speed in the numerical model. (B) Correlation length as a function of the cell speed in the numerical model. Cell speed in both cases is calculated in the flat condition from the net displacement over 10 Monte Carlo Time-Steps (MTS) and averaged over 2300 cells.

Consistent with a volume exclusion mechanism and our in silico results (Figure 4.11C), our experimental data show that both correlation length (Figure 4.13A) and signal propagation distance (Figure 4.6F and Figure 4.13B) correlate with cell density. Depending on the properties of the crowded particles, the length over which correlated behavior extends is also predicted to depend on particle speed. These trends are reproduced in the model, since larger cell velocities arise due to larger motile forces, which are also responsible for a decrease in cell-cell coordination (Figure 4.14). Experimentally, we also observe this dependence: our data show a statistically significant inverse relationship between correlation length and cell speed (Figure 4.13C), where faster objects show correlated behavior over a shorter range. Similarly, propagation distance is inversely proportional to cell speed (Figure 4.13D). Since correlation length and propagation length both show similar dependencies on object size (cell area) and object speed, we assessed the correlation of these parameters in both our in silico and in vitro data. Both our model (Figure 4.11D) and our experimental data (Figure 4.13E) show a statistically significant correlation between correlation length and propagation distance, providing further evidence that the volume exclusion mechanism underlying cell streaming also determines signal propagation distance in this context.

Taken together, our data suggests that when cells are in constrained space, tension-based forces at junctions, which are typically used to explain cell-cell cooperation during group migration, are not in fact a dominant factor in determining cooperative behaviors such as cell streaming and propagation of guidance signals. The spatial range over which cooperative behaviors such as
streaming and signal propagation extend falls within a limited range (6-9 cells) over a wide range of experimental conditions. This is consistent with a model in which intrinsic physical properties of the cells are critical parameters that impact cell migration, but are essentially unchangeable in typical culture conditions. For example, altering cell viscosity (which impacts cell compressibility) could change collective migration, but would also disrupt the viability and confluence of the cell sheet. Even cell area, which we found impacts cooperation, can only vary within a limited range while maintaining confluence. A key challenge for the future will be to better understand the underlying mechanism driving stream formation in dense cell sheets and hence determine what cellular parameters, in addition to area, dictate the number of cells that participate in a particular stream. Although our work provides a better understanding of the factors that affect how cells cooperate during group migration in a constrained and textured space, a complete understanding of cell cooperation is still required to provide important tools for engineering tissue morphogenesis for regenerative medicine applications and to provide insight into tissue formation in the developing embryo.

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4.9 References


Chapter 5
A Scratch Wound Assay for the Characterisation of the Temporal and Spatial Dynamics of Collective Cell Migration

5.1 Commentary

In this work, we propose a cell-tracking based analysis method for the classical wound healing assay, and provide evidence that the traditional end-point analysis is unable to capture the temporal and spatial dynamics of cell migration within this system. We present a rationale for why more thorough analysis is necessary: a variety of cell-level changes in behaviour, each of which may be related to different molecular changes, can potentially result in tissue-level phenotypes that are similar. Understanding the cell-level changes in behaviour can provide guidance as to potential molecular pathways involved in any observed phenotypes.

Despite its simplicity, the scratch wound assay is remarkably complex in terms of signalling: cells are exposed to a variety of chemical and mechanical guidance signals, the distribution of which depends on both time and space. While the proposed analysis method is unable to ascertain the contributions of different guidance mechanisms, the temporal and spatial resolution it provides can differentiate between signals that propagate in a time- and space-dependent manner (such as the chemical signals released by the act of wounding or the mechanical signal driven by changes in cell density as the cells move into the denuded area) and signals that are constant (such as topographic guidance, haptotactic signals that do not change over time or random migration). As such, this analysis improves upon existing analysis methods that conflate all changes in wound front movement (including those that arise from cell expansion) into one single output. However, it is worth noting that this method requires more complex equipment than the traditional method (as cells must be imaged live), and is much more time-consuming to analyse.

5.2 Introduction

The scratch wound assay, in which a confluent sheet of cells is damaged and the recovery quantified, is a common and classic assay for cell migration. It is often used in studies of genes\textsuperscript{1} (particularly oncogenes\textsuperscript{2}) or cancer drugs and small molecules\textsuperscript{3,4} that impact cell migration.
because it allows for easy and inexpensive evaluation of how particular modifications or compounds affect the migratory abilities of cells.

The current analysis methods for the scratch wound assay focus only on acquiring tissue-level information about the rate at which the sheet front moves to repopulate a denuded area. While this makes analysis (and, if used as an endpoint assay, data acquisition as well) simple, it fails to take advantage of how powerful the assay truly is. In particular, any reported changes in migration do not provide information about changes in migratory behavior that occur at the individual cell level.

A change in wound closure rate can arise from a variety of changes in individual cell behaviour: increased cell speed, increased persistence and increased directedness could all independently increase the wound closure rate\(^5\). The movement of the edge of the wound does not capture these differences, and given that each of these variables appear to be controlled by a different set of proteins\(^6\), scratch wound experiments analysed in this way miss valuable biological information.

Additionally, despite the fact that the behavior of advancing cell sheets in the scratch assay is characterised by both temporal and spatial heterogeneity, the assay is most often analysed by looking at the movement of the sheet front between two time points\(^7\). This ignores not only the differences that take place within the cell sheet, but also the temporal sequence of events that occurs as the cells respond to the wound.

Here, we describe a more complex method for analysing cell behaviour during scratch wound closure by thoroughly characterising individual cell behaviour across the sheet and over all experimental time by integrating the standard scratch wound assay with an individual cell tracking assay. While it involves more complex data collection and analysis, it enables better understanding of how individual cellular behaviour contributes to scratch wound closure and as such can be more useful to understand how cell migration changes depending on inputs.

### 5.3 Materials and Methods

**Cell Culture**

Human umbilical vein endothelial cells (HUVEC) (Lonza, USA) were grown in Endothelial Basal Media (EBM-2) supplemented with the EGM-2 Bullet Kit (Lonza) and used within 8
passages. HUVEC were transduced with lentiviral constructs for Vangl1 or GFP. ARPE-19 were grown in Dulbecco’s Modified Eagle’s Medium/nutrient F-12 (DMEM/F12) (Invitrogen, Canada) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (VWR, Canada). All cells were maintained in a humidified atmosphere at 37°C and 5% CO₂.

**Wound Healing Assays**

The wells of Vision Plate 24 (4titude Ltd., United Kingdom) (for HUVEC) or 24-well tissue culture-treated polystyrene plates (BD, USA) (ARPE-19) were coated with 314µg/mL PureCol (Advanced Biomatrix, USA) for 1 hour. After rinsing the wells with PBS, cells seeded at a density of 84,000 cells/cm² (HUVEC) or 90,000 cells/cm² (ARPE-19) and allowed to settle and attach for 24 hours. The cells were then stained with 500ng/mL Hoechst 33342 (Invitrogen) and 5µg/mL CellMask membrane stain (Invitrogen) for 30 minutes and rinsed with PBS (VWR) once. Wounds were scratched in each well using a P10 tip, after which the wells were rinsed with PBS once more. Plates were kept warm by placing them on a heat-retaining surface to minimize thermal expansion and contraction effects. The plates were then placed in an ImageXpress Micro high-content screening microscope with a humidified live-cell imaging module (Molecular Devices, USA) kept at 37°C and sparged with 5% CO₂, and imaged every 20 minutes for 6 h (HUVEC) or 23 h (ARPE-19).

**Cell Tracking and Position-Based Analysis of Cell Migration**

Nuclear images were used to generate cell tracking data using the Multi-Dimensional Motion Analysis application module in MetaXpress software (Molecular Devices). This algorithm uses nuclear shape and stain intensity to distinguish between nuclei, identify them in subsequent frames, and consequently track nuclear motion across images.
**Figure 5.1 – The Proposed Analysis Method Separates Cell Behaviour According to Location:** A) Schematic of slicing strategy. The location of the wound is marked using a straight line. The first slice is larger than the others to ensure all cells at the leading edge are included despite irregularities at the wound edge. B) Migration tracks of all cells in a well (left) and tracks from the first five slices from the bottom of wound (right). By separating the migration behavior by slice, it becomes noticeable that the bright yellow patch located at approximately the centre of the left image includes cells from not just the front of the wound but from several slices back.

The tracking data and the position of the wound were then passed on to a custom-made MATLAB program that defines slices, sized according to user input, starting at the wound site and moving back into the sheet (Figure 5.1A). A cell’s starting position determines the slice it belongs to. Migration parameters were calculated individually for each cell, and then grouped or averaged by slice to obtain spatial differences in migration behaviour. The MATLAB program was also designed to plot cell tracks for each slice separately (Figure 5.1B) and to calculate the change in migration behavior over time by examining each time frame individually.
5.4 Results and Discussion

5.4.1 Metrics to Characterise the Migratory Behaviour of Cells in the Wound Healing Assay

Figure 5.2 – Metrics to Characterise Population-wide and Individual Cell Migration-Specific Scratch Wound Closure Response: A) The traditional scratch wound analysis measures the change in denuded area over time (typically between two time-points). B) The forward component of the sum of the migration vectors of each cell over time are analogous to the movement of the wound front. C) Differences in movement in the forward direction...
may arise from different migration phenotypes. i) A control cell moves farther than: ii) A cell that moves at the same angles as the control cell, but at much lower speed; iii) a cell that moves at the same speed as the control cell, but turns more sharply and often; and iv) a cell that moves at the same speed as the control cell but is not directed to move forward. Notice that the forward displacement of ii-iv is the same despite differences in behaviour. D) Schematic of speed and persistence measurements used in this analysis. E) The change in the persistence metric over time is calculated by looking at the change in net displacement at each time frame (dark blue, light green, purple, light blue, dark green) over the total distance travelled up to that time frame (the sum of the red arrows at each point). F) Angular metrics used in this analysis: $\Theta$ describes the angle of migration with respect to an axis perpendicular to the wound. $\Theta$ equals 0 when the cell moves straight into the wound. $\alpha$ is the change in angle between the migration direction in subsequent time frames. G) The change in $\alpha$ is calculated over time by changing the size of the analysis window. Notice that in window size = 1, $\alpha$ is calculated at all timepoints, while some of the time points are not used for window size = 2 or 3.

The traditional scratch wound assay analysis measures the change in area covered by cells over time (Figure 5.2A). In instances such as re-epithelialization after injury, the movement of the sheet front is important because it characterises how quickly full barrier function can be recovered, which depends on the movement of the whole sheet and not that of the individual cells. The analysis presented here characterises the same behavior by quantifying the net migration in the direction perpendicular to the wound of each cell (Figure 5.2B). However, the traditional analysis is unable to parse how cell-level behavioral changes contribute to tissue-level changes in the movement of the front of the wound.

Our analysis uses cell tracking to gather information at the individual cell level, and calculate cell migration parameters like cell speed and migration angle for each cell in the wounded sheet. We chose to do this because it allows us to better understand how individual cell migration dynamics impact wound closure rate, making the results of the wound healing assay more informative. This is important because tissue-level analysis ignores how different behaviors can all lead to the same outcome (Figure 5.2C). The example cell on the left (Figure 5.2Ci) has moved forward more than the three example cells on the right (Figure 5.2Cii-iv), which all have the same amount of net forward movement. However, the reasons for this decrease are very different: shorter steps (lower migration speed) (Figure 5.2Cii), lower directional persistence characterised by increased turning frequency/angle (Figure 5.2Ciii), and reduced attraction leading to migration at a less effective angle for wound closure (Figure 5.2Civ).

Because differences in migration could go undetected if gene modifications or drug treatments simultaneously but oppositely affected two or more of: migration speed, time needed to achieve polarization/onset of migration, persistence time, and chemotactic response/directed migration, it is important to use a cell-based approach to analyse a scratch wound assay. Cdc42, for example,
is known to affect the polarization of cells; it is critical to the reorientation of the microtubule
organizing center (MTOC), which is essential for cell migration to occur. An experiment in
which defects in directed migration were observed would warrant further examining (by live-
imaging or immunofluorescent staining) whether Cdc42 levels or localization were altered.
Characterizing behavioural changes at the cell level could provide some instruction to understand
which migration-related components may be affected in any particular experiment and as such,
help provide avenue for further exploration.

In addition to the forward component of migration, we have incorporated into our analysis a
number of common and innovative cell migration metrics that we believe are most informative in
terms of cellular behavior. These include:

- Cell speed (Figure 5.2D) refers to the rate at which a cell moves, and is calculated by
dividing the distance moved by a cell in a certain time frame and dividing by the length of
that time frame. Cell speed can be affected by many different variables, including
cytoskeletal polymerisation rates, cell contractility, and focal adhesion dynamics.

- Cell persistence, which is also referred to as directionality ratio or directional index,
quantifies the effectiveness of cells in moving away from their point of origin by dividing
net displacement by total distance travelled and considering the evolution of this ratio over
time (Figure 5.2D-E). This can depend on the susceptibility of the cells to respond to
directional signals, lamellipodial persistence and stability, and cell-cell coordination,
among others.

- Angular metrics can be very helpful to understand whether a cell’s migration is being
affected by external signals or is no different from random motility (Figure 5.2F). Migrat
angle ($\Theta$) is affected by cell-cell coordination, intrinsic cell chirality, and each cell’s response to the chemical and physical cues that arise from the act of wounding the cell sheet. We have devised a new metric, $\alpha$, that measures the change in migration angle between two evenly spaced time windows (Figure 5.2F-G) (ie. The turning behaviour of the cells between two time windows). By expanding the size of those windows, we can estimate how long these cells will continue to migrate in the same general direction. This relates to persistence time, or how often a cell changes direction. Like migration angle, it can be affected by cell-cell coordination and cell chirality, but is also related to lamellipodial dynamics.
A major advantage of our proposed solution is that, because it relies on cell positional data acquired repeatedly across experimental time, additional metrics may be added post-hoc.

5.4.2 Characterizing Scratch Wound Healing Dynamics

Figure 5.3 – Cell Migration Dynamics at the Wound Front Demonstrate that Migration Changes Over Time:

A) The forward component of cell migration in the first slice increased over the first six hours of migration analysis, which corresponded primarily to cell expansion rather than migration. Forward movement rate then steadied until the sheet fronts met each other, at which point it decreased. B) The behaviour of the cells during the first six hours contributed heavily to analysis of cell front movement, which indicates that the choice of endpoint of the experiment is important. C) Cell speed at the wound front over time: Cell migration speed increases within the first 6 hours of the experiment, then plateaus, and decreases slightly when the wound front meet each other. It is worth noting that the pattern of forward movement (Figure 5.3A) cannot be explained exclusively by changes in cell speed, which followed slightly different patterns particularly at the end of the experiment. This supports our hypothesis that more detailed analysis of migration behaviours is necessary to understand how cell behaviour affect wound closure rates. D) The persistence metric changed rapidly within the first six hours, but remained steady for the rest of the experiment, though a small drop did occur in the last few hours. E) The rate of decay of persistence was much higher when the first six hours, which correspond to cell expansion rather than migration, were taken into consideration (red line) than when the analysis started after expansion was completed (blue line). F) The majority of the cells at front migrated toward the denuded area (blue line). However, the proportion of cells that was directed forward (45° from the vertical – red line and 30° from the vertical – grey line) was variable, and larger during the expansion process (first six hours) than during active migration. This proportion also decreased rapidly once the wound fronts come into contact with each other. G) The percentage of cells which migrated within 30° of the same
direction as in the previous time window was never lower than 50%, suggesting the cells are very persistent. Error bars are standard error of the mean (n=4).

The process of sheet migration after a scratch has long been known to take place in stages, with cell behaviour changing over time. Endothelial cell experiments have shown that the first six hours after wounding are primarily characterised by cell expansion and reorganization of the cytoskeleton; cells do not begin migrating in earnest until 6h post-wounding\textsuperscript{16}.

We first plotted the average size of the forward component of migration for cells in the first slice and found that the slope of the resulting line was not constant (Figure 5.3A). In the first several hours, the forward component of cell motion (a proxy measure for front displacement) increased over time (increasing slope), before steadying at approximately hour six, which agrees with the previously reported endothelial cell data\textsuperscript{16}. Since the first six hours are characterised by changes in cell morphology rather than active migration, we plotted the ratio of the forward motion up to that point to total forward movement to illustrate how the choice of endpoint can impact the results significantly depending on cell behavior (Figure 5.3B). For at least 7 hours, the contribution of cell expansion impacts the wound healing rate more than that of cell migration.

We then plotted the migration speed of the first slice over time (Figure 5.3C) to determine whether the observed changes in front migration rate could be explained exclusively by changes in migration speed. Comparing Figure 5.3A to Figure 5.3C demonstrates that this is not the case: the forward movement graph plateaus earlier than the cell speed one; the former also has a much more drastic drop in the final hours of the experiment than the latter does, which indicates that at the end of the experiment, cells continue to migrate but no longer move forward (which is consistent with the sheet edges coming into contact with each other). If all changes in migration could be explained by cell speed, the graphs would look exactly the same and differ only in scaling.

We noticed that after approximately six hours, speed and persistence of the cells at the front stabilized, as shown by the plateaus in Figure 5.3C-D. We also noticed that these stable levels (particularly in cell speed) only changed again once the two cell fronts ran into each other (between 18 and 19 hours in these experiments).

Since cell persistence is a measure of how effective migration is, we reanalysed the persistence data by setting the start point after the cell migration speed has stabilized (t=6h), likely because
cell expansion is complete and cells are actively migrating (Figure 5.3E). That the values of persistence were much higher was not surprising, but it reinforced the need for a dynamic analysis since it further illustrated the point that in the scratch wound assay, the choices of start and end-point are non-trivial and can affect the results significantly.

By examining how migration parameters such as migration speed, cell persistence, and migration angle change over time, the analysis proposed here is less reliant on an appropriate choice of endpoint and overcomes issues with over- or under-representing certain differences in cell behaviour: if, for example, a particular modification were to result in cells having delayed polarization but increased speed, an endpoint assay may show no difference in wound healing, missing that the cells had a delayed onset of migration, but overcame that by moving faster.

We then considered the angular metrics described in Figure 5.2F-G, $\Theta$ (which describes the direction of motion of each cell) and $\alpha$ (the angle between the direction of motion in adjacent timeframes), which also provide us with valuable information about how the cells’ behavior changes over time. Nearly 100% of the cells migrate in the direction of the wound (within 90° of the direction of the wound) at the front of the sheet (Figure 5.3F, blue line). There is a very slight increase in this proportion in the first 6 hours, consistent with the results from the speed and persistence data that suggest that the migratory behaviour of the cells is different in earlier timepoints. Examining the percentage of cells moving at a 45° (red line) or 30° (grey line) angle from the vertical shows that the expansive movement at the beginning of the experiment appears to be very directed. Since the cells are not actively migrating, this is reasonable as cells would be more likely to expand toward the region of free space instead of toward their neighbours. The proportion of the cells moving directedly decreased moderately and then plateaus during the migratory portion of the experiment, until the time when the wound edges are getting close to touching. That this angular metric decreases before the wound edges come into contact with each other suggests there may be some communication between the cells that make up the sheet fronts.

The $\alpha$ metric, which characterises how long it takes for a cell to change directions, shows that the cells at the front of the wound move in a persistent manner (Figure 5.3G). 73.4% ± 1.9% of the cells migrate in the same direction as in the previous time frame, and 61.2% ± 1.3% maintain their direction of migration for an hour; most remarkably, 46.8% ± 7.8% maintain their general
direction of migration throughout the experiment, which indicates cells respond very strongly to the directionality signals that are provided by the wound.

5.4.3 Capturing Spatial Heterogeneity and Dynamics

**Figure 5.4 – The Migratory Behaviour of the Cells Depends on their Distance from the Wound, Their Phenotype, and Elapsed Time Since Wounding:**

A) The dynamics of forward displacement depend on distance from the wound. Cells that were located farther from the wound edge moved forward less than counterparts that were closer. Additionally, the time it took for their forward migration to plateau also increased with distance from the wound.

B) The propagation of the wound signals depends on cell phenotype: Vangl1-overexpressing HUVEC had a positive forward component of movement (which decays to zero in random migration) up to 600μm away from the wound, while control cells decayed to zero in 300μm.

C) Cells also move less efficiently farther away from the wound. The rate of decay of persistence increased with distance, while the plateau level decreased.

D) The evolution of migration angle also depends on distance from the wound. While more cells migrated in a directed fashion closer to the wound in earlier timepoints, the proportion increased to be nearly equal after 10h. Error bars are standard error of the mean (n=4).

Others have shown that there is significant spatial and temporal heterogeneity within the advancing monolayer after it is scratched, where this migratory behaviour propagates back spatially as time proceeds\(^ {17} \). A graph of forward migration versus time (Figure 5.4A) for selected slices shows how cell migration propagates from the front of the wound, with this distance
increasing for all slices in a time- and distance-dependent manner: At the start of the experiment, the median forward migration is lower the farther from the wound (Figure 5.4A). The graphs also plateau later the farther the slice is from the edge (compare Slice 1 to 5, and to 10, which appears to be reaching its plateau value at the conclusion of the experiment).

Others have shown that migratory responses propagate back from the wound front, and have suggested that there is a limit to how far this propagation goes, beyond which cells no longer sense the wound\(^1\). In an undamaged sheet, or far enough from the wound that the cells are not aware that a wound exists, migration is random, and as such, the average forward migration is zero. Data from Vangl1-modified HUVEC experiments indicate the propagation of the wound signals may depend on cell phenotype (Figure 5.4B): Vangl1-overexpressssing HUVEC have a positive forward migration up to approximately 600\(\mu\)m from the wound edge compared to 300 \(\mu\)m for control cells.

The persistence also changes in a distance-dependent manner, plateauing at lower levels the farther from the wound (Figure 5.4C). Given that this number relates to cell migration efficiency, it is not unexpected that it is highest at the front of the sheet, where cells have a free edge, and lower when cells are surrounded by neighbours. The progression of migration angle over time also has a critical space-dependent component. As the influence of the wound propagates back, the migration angle of the cells changes, reflecting increases in directed, rather than random, migration (Figure 5.4D). The proportion of cells that migrate in a directed fashion increases over time. The time at which the proportion of the cells which migrate within a 30° or 45° degrees of the vertical (or perpendicular to the wound) begins to increase reflects the point at which cells within a specific slice start to feel the effects of the wound. For the most part, this time increases the farther from the wound a slice is located, as evidenced by the differences between the blue, red and gray lines in Figure 5.4D. The yellow line, however, corresponds to the point where all of the cells in the analysed portion of the sheet are being affected by the wound, and as such does not show as much spatial variation.

### 5.5 Conclusions

We have presented here a thorough method for analyzing scratch wound assays based on cell tracking. While this new method necessitates a more complex experimental setup (particularly the ability to live image multiple samples concurrently) and increases the time and effort
required for data analysis, its added complexity is reflected in how much more additional information it provides. Dissecting how cellular behaviour depends both on space and time in this assay allows for much more complete and useful information to be gathered from this classic assay. One of the main reasons this assay is so popular is because it is easy to perform while allowing parallel analysis of multiple samples, neither of which change in this method. By increasing the amount and specificity of the data that this assay provides, our analysis makes a powerful cell biology tool even more useful for researchers.

5.6 References


6.1 Commentary

This work is currently being prepared for peer review and publication with co-authors Jianxun Han, Jonathan Brunetti, and Alison McGuigan (corresponding author). Author contributions: Study concept and design: CL, JH, JB, APM. Migration experiments and analysis of migration in shear flow experiments: CL, APM. Shear flow experiments and analysis: JH, JB, APM. Manuscript preparation: CL, JH, APM.

Figure 6.1 - The migration behaviour of a cell is affected by multiple cues it receives from its environment: In this chapter, we explore how the migratory behaviour of endothelial cells depends on a combination of cues, and specifically how Planar Cell Polarity (PCP) signalling affects the impact of these cues on cell migration. We consider the complex milieu of signals and behaviours that arise as a consequence of scratching a cell monolayer (which include chemical, mechanical, and electrical signals released/cause by the act of scratching; elongation and polarization of cells at the wound front; and the introduction of free space/removal of spatial constraints), and provide them in separate assays in order to understand how these individual cues interact with the PCP pathway.

The Planar Cell Polarity (PCP) signalling pathway is, as mentioned earlier, a known player in coordinating cell behaviours across tissues. As its name indicates, it has been implicated in particular in establishing planar polarization (ie. Polarization in the plane of the tissue) in a number of different systems. However, how the PCP signalling pathway determines the correct
axis for polarization is not yet understood. A common hypothesis suggests an as-yet-unknown global polarizing signal that the PCP signal amplifies through feedback between its components.

Given the established but not always consistent role of the PCP pathway in guiding cell behaviour, we wanted to explore how it would interact with other guidance mechanisms (Figure 6.1) in the guidance of cell migration, and particularly, whether specific contexts are necessary for PCP to affect cell migration. We chose to use endothelial cells for that purpose for several reasons: firstly, the collective migration of endothelial cells has been well-characterised by others, and is known to depend strongly on directionality mechanisms. Secondly, the migration of endothelial cells in vivo is critical during development and after birth, for the healing of injuries and in pathological conditions, so understanding what governs their migration is important. Thirdly, endothelial cells are known to respond to a variety of guidance signals including topographic cues, chemical signals, and mechanical forces, which makes them an attractive model for understanding how guidance mechanisms interact.

The most important finding in this chapter is that, while the PCP signalling pathway protein Vangl1 does affect the migration of endothelial cells, it only does so in the scratch wound assay, in a response that appears to be guided by damage signals from the scratching action; further, the effect that Vangl1 appears to have is that cells move in a more directed fashion (though slower), and that cells even farther from the wound migrate in a directed fashion, which suggests the propagation distance of the damage signals may be longer. That a member of the PCP family affects a polarized behaviour, such as directed migration, is not surprising, but the importance of context has not, to our knowledge, been explored before. The interaction between damage signals and PCP signalling and the importance of context in migration assays were both highlighted in this chapter, and will hopefully provide fruitful avenues of investigation in the future.

6.2 Abstract
The endothelial cells (EC) that make up the vascular endothelium migrate and reorganize during a number of physiological and pathological processes. The mechanisms underlying how EC coordinate their behaviour during these collective migration and tissue re-organization processes are not well understood. A possible regulator of coordinated migration in EC is the Planar Cell Polarity (PCP) signalling pathway, which is known to coordinate a number of cellular behaviours
in a range of tissues across species. Here, we explore the impact of the planar cell polarity protein Vangl1 on the migration of EC using a variety of single cell and collective cell migration assays. We find that modulating Vangl affects collective but not single cell EC migration and more specifically that Vangl1 modification only impacts collective cell migration in the context of a scratch wound assay, but not during re-organization of confluent sheets, cell re-organization on exposure to shear flow, or during group movement into free space in the absence of a wound. Furthermore, we show that increased wound closure rates in Vangl1 over-expressing cells result not from increased cell movement but from increased directedness of cells proximal to the wound edge as a result of less frequent and smaller cell turns. Our study highlights the importance of context in the study of migratory behavior, and also provides evidence that Vangl1 plays a role in coordinating directed EC migration specifically.

6.3 Insight statement

Endothelial cells rearrange as a collective group during a number of tissue remodeling processes but the mechanism of cell coordination during these processes is not well understood. The planar cell polarity signalling (PCP) pathway coordinates collective cell behaviours in a number of tissues. Here, we explore the influence of the PCP pathway on EC wound healing using live imaging and custom-built image analysis algorithms that enable us to stratify the behavior of individual cells within cell groups. Our approach provides cell-level rather than tissue-level data, allowing deeper understanding of how PCP signalling affects individual cell migration within groups, further evidence that PCP signalling impacts EC reorganization, and clues to better understand the molecular mechanisms underlying these effects in the future.

6.4 Introduction

Endothelium lines the vascular structures throughout the body and is highly dynamic both during development and in adult tissues\(^1\). The endothelial cells (EC) that make up the vascular endothelium must effectively adapt and reorganize to accommodate assault by cells of the immune system\(^2\), adapt to changes in blood flow due to exercise or disease\(^3\), enable vessel wall recovery in the event of damage\(^4\), and generate new vessels via angiogenic sprouting and vascular pruning\(^5\)\(^-\)\(^7\). Essential to the endothelium’s adaptability is the ability of EC to migrate and rearrange\(^8\)\(^-\)\(^10\) not just as individual cells but as a collective coordinated group, termed collective
cell migration\textsuperscript{11,12}. Collective cell migration differ from single cell migration in that it requires that cells in the group remain connected to each other through cell-cell junctions and coordinate their individual migratory behaviours\textsuperscript{12}. The mechanisms underlying how EC coordinate their behaviour during collective migration and tissue re-organization are not well understood\textsuperscript{13,14}. Understanding these mechanisms, however, could suggest strategies for manipulation of collective migration to enhance angiogenesis or wound healing for tissue regeneration or to inhibit angiogenesis in diseases such as cancer. Furthermore, understanding these mechanisms in EC may provide insight into the collective migration of other cell types, particularly those which share an epithelial origin with the endothelium.

Two collective processes involving EC sheet re-organization that have been extensively studied are the coordinated alignment of EC when exposed to shear flow\textsuperscript{15,16} and wound closure after the introduction of a scratch into an endothelial sheet\textsuperscript{17-19}. In the first of these models, cell alignment in response to shear flow, many possible molecules/structures, often located on the cell membrane, including ion channels\textsuperscript{20}, caveolae\textsuperscript{21}, integrins\textsuperscript{22}, the glycocalyx\textsuperscript{23}, PECAM-1/VE-Cadherin/VEGFR2 complexes\textsuperscript{24}, and primary cilia\textsuperscript{25} have been proposed to be involved in the sensing and regulation of EC response to the mechanical stress signal. Furthermore, while it is not known how the input of these mechanosensors is coordinated, it has been shown that the integrated signal from their downstream signalling pathways conveys on small GTPases, Rac1 and RhoA\textsuperscript{26-29}, which in turn regulate the remodeling of microtubules and actin fibres to induce a planar polarized and aligned cell morphology in the cell sheet.

The second of these models, scratch wound closure, has been thoroughly characterised from a phenomenological standpoint\textsuperscript{30}: the introduction of a wound into an EC sheet triggers actin reorganization (specifically, the breakdown of the dense peripheral band and the extension of lamellipodia) and partial cell-cell detachment at the wound edge within two hours. Between two and four hours after wounding, cells expand laterally and reorganize their actin to align parallel to the wound; additionally, most of the microtubule organizing centers (MTOC) polarize and become localized towards the front of the nucleus\textsuperscript{31} and to the wound edge. Approximately six to eight hours after wounding, intracellular actin has aligned perpendicular to the wound, and active cell migration begins. Both microtubule and actin networks are essential to wound closure and disruption of either results in no or reduced wound closure\textsuperscript{18,32}. A number of genetic regulators of this wound closure process have also been identified\textsuperscript{19}. Specifically, a modular model has
been proposed in which sets of genes independently affect proliferation/cell density, individual cell movement (e.g. RhoA), directed migration (e.g. PTEN and FGFR1), and coordination between cells in the sheet (e.g. α-catenin). The directedness and coordination of EC migration, at least in the context of growth factors being present during wound closure, are thought to be dependent on the cell-cell coordination module (as well as individual cell motility) and governed in part by leader cells (which may be controlled by Ca\(^{2+}\) signalling\(^{33}\)) that respond to the growth factor signal and help direct follower cells. The directedness and coordination of EC migration have also been shown to depend on other factors, such as substrate stiffness\(^{34}\). Furthermore, evidence suggests that the integration of these signals hinges on the Rho family of small GTPases\(^{35-37}\), but how all these potential mechanisms result in coordinated EC migration is not well understood.

A possible regulator of coordinated migration in EC is the Planar Cell Polarity (PCP) signalling pathway, which is known to coordinate a number of cellular behaviours in a range of tissues across species\(^{38-42}\). The PCP pathway was first identified through its role in the coordinated localization of actin hairs in the \textit{Drosophila} wing\(^{43}\) and has since been implicated in a variety of coordinated behaviours, including convergent extension\(^{44-47}\), the orientation of stereocilia in the mouse ear\(^{48}\) and of mammalian hair follicles\(^{49}\), the rotation of ommatidia in the \textit{Drosophila} eye\(^{50}\), oriented cell division and cell intercalation in the kidney\(^{51-53}\), and the polarized alignment of motile cilia in the trachea\(^{54}\). In mammals, the major core components of the PCP signalling pathway are Frizzled (Fz), Vang-like protein (Vangl), Dishevelled (Dvl), Prickle (Pk), Celsr (Celsr), and Diversin (ankrd6)\(^{42}\). Manipulation of these core PCP proteins has been shown to impact the migration of numerous cells including epithelial cells of the anterior visceral endoderm (AVE)\(^{55}\), cancer cells\(^{56-58}\), facial brachiomotor neurons\(^{59-61}\), neural crest cells\(^{62, 63}\), and endothelial cells\(^{64-68}\), but most studies have not differentiated between effects on single cells versus movement in collective groups of cells. The general trends suggest inactive Dvl, inhibition of Fz, or knockdown of Wnt5a (the ligand that binds to the Fz receptor) suppress migration in ECs\(^{65, 66, 69-71}\). Over-expression of constitutively active Daam1 also inhibits EC migration\(^{66}\). Results for Vangl are more conflicting: In some systems, over-expression increased migration\(^{72, 73}\) and knockdown reduced migration\(^{74}\), while in others, over-expression decreased migration\(^{57}\) and knockdown increased migration\(^{57}\) or had no effect\(^{57, 72}\), depending on the assay (single versus collective cell migration). No Vangl studies, however, have been performed in EC.
and the inconsistency between studies in other cells suggests that different PCP proteins influence cell migration differently, that different cells (tumor or normal) may behave differently, and that the context of the cell migration assay may also be important. The downstream mechanism by which PCP signalling impacts the cell migration machinery is not well understood but is thought to involve Rho/Rac and consequently, remodeling of microtubules and actin fibres. Given that the PCP pathway has been shown to regulate several planar polarized cell behaviors involving small GTPases, which are also critical for the planar polarized cell morphology of ECs in response to unidirectional shear stress, and that the PCP pathway has also been shown to impact EC migration, we set out to investigate if the PCP pathway is involved in collective cell migration in EC.

Here, we specifically explore the impact of the planar cell polarity protein Vangl1 on the migration of EC (both single cell and collective). Among the core PCP proteins, we chose to focus on Vangl proteins specifically for a number of reasons. Firstly, among the transmembrane core PCP proteins, Vang-like protein has the least (two) mammalian homologs compared to three Celsr proteins and ten Fz homologs. This reduces the challenge of potential functional compensation among homologs. Secondly, Vangl mutants suffer from severe cardiovascular development defects, which warrant considering whether Vangl proteins affect endothelial cell migration. Furthermore, Vangl2 plays an active role in regulating cell junctions and physically interacts with Rac1 in epithelial cells; both cell junctions and Rac are known to be important in the coordination of collective cell migration. Lastly, and most importantly, though other PCP proteins have been shown to affect endothelial cell migration, no studies have been reported to date on the effect of Vangl on the migration of endothelial cells. Using a number of different cell migration assays, we find that modulating Vangl affects collective but not single cell EC migration. Furthermore, we find that Vangl1 modification only impacts collective cell migration in the context of a scratch wound assay, but not during re-organization of confluent sheets, cell re-organization on exposure to shear flow, or during group movement into free space in the absence of a wound. We also show that increased wound closure rates in Vangl1 over-expressing cells result not from increased cell movement but from increased directedness of cells proximal to the wound edge as a result of less frequent and smaller cell turns, consistent with the molecular pathways that are associated with PCP signalling. Our study
highlights the importance of context in the study of migratory behavior, and also provides evidence that Vangl1 plays a role in coordinating directed EC migration specifically.

6.5 Materials and Methods

Cell Culture

Pooled human umbilical vein endothelial cells (HUVEC) (Lonza, USA) were cultured in Endothelial Basal Medium-2 (EBM-2) supplemented with EGM-2 BulletKit (EGM-2) (Lonza) at 37°C and 5% CO2. P0 HUVEC were plated, allowed to grow to confluence, and then passaged, aliquoted, and frozen in 90% Fetal Bovine Serum (FBS) (VWR, Canada) /10% Dimethylsulfoxide (DMSO) (BioShop, Canada). Cells were passaged by removing the medium, rinsing with 1x phosphate buffer saline without calcium or magnesium (PBS) (VWR, Canada), and then adding 0.25% Trypsin EDTA (Sigma-Aldrich, Canada). This trypsin was removed immediately, and the cells incubated for 5 minutes at 37°C and 5% CO2. Fresh medium was added to stop trypsinization, and the suspension centrifuged to form a pellet. After decanting the medium, the pellet was resuspended in EGM-2 to the appropriate concentration. HUVEC were passaged every 5-7 days at approximately 1:5 ratios, and used within 8 passages.

Lentiviral production

3.5 x 10^6 HEK-293T/17 virus packaging cells were seeded on 100 mm tissue culture treated plates (BD Falcon, Canada) in Dulbecco’s Modified Eagle Medium (DMEM) (VWR, Canada) supplemented with 10% FBS and 0.1% Penicillin/Streptomycin (VWR, Canada). To produce lentiviral vectors, expression and packaging plasmids were co-transfected into the cells using Fugene6 (Roche, Mannheim, Germany) with a ratio of 10:9:1 for expression plasmid, psPAX2 and pMD2.G respectively. Virus particles were then harvested in DMEM supplemented with 1.28% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, Canada) and 1% Penicillin/Streptomycin at 48 h and 72 h after transfection and were concentrated by Amicon® Ultra-15 centrifugal filter unit with Ultracel-50 membrane (Millipore, Billerica, MA). Viral aliquots were deep frozen until use.
**Lentiviral transduction**

We optimized the transduction protocol based on GFP control lentivirus and non-target shRNA control lentivirus, and found that a transduction efficiency of 60-80% was ideal as transduction with efficiencies higher than 80% were always associated with decreased cell growth and changes in cell morphology. To transduce HUVEC, 1 x 10^6 cells were seeded into a 100 mm cell culture dish and allowed to attach overnight before incubation with appropriate amounts of lentiviral vectors and 25 µg/ml of polybrene (Sigma-Aldrich) in a 5-ml volume for 18-24 h. Transduced HUVEC were then purified by fluorescence-activated cell sorting (FACS, for GFP or GFP-Vangl1 lentiviral transduction) or puromycin selection (for shRNA lentiviral transduction).

**Fluorescence Activated Cell Sorting (FACS)**

HUVEC expressing GFP or GFP-Vangl1 were resuspended in PBS supplemented with 10% FBS. The cells were sorted at the Princess Margaret Hospital Flow Cytometry facility. GFP fluorescence was excited with an argon laser and detected using a 530/30 nm band-pass filter in FL1 channel. The front and side scatter were used to exclude any dead cells. Fluorescent cells were selected from the live population, and the bottom 5% and between 5 and 10% of the top population were excluded. Cells were allowed to recover for at least 5 days prior to use.

**Reverse transcription-coupled PCR (RT-PCR) & quantitative PCR (qPCR)**

Total cellular RNA was extracted using an RNeasy® Mini Kit (Qiagen, Germany), homogenized using the QIAshredder (Qiagen) and treated with RNase-free DNase I (Thermo Scientific, USA) to remove any possible genomic DNA contamination. cDNA was synthesized using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, USA). To detect the presence of Vangl1 and Vangl2 transcripts, conventional PCR of 35 amplification cycles was performed using Taq DNA polymerase (Sigma-Aldrich) with at least two different pairs of primers (listed in Table II.1 – Appendix II). The PCR products were then separated onto 2% agarose (BioShop) gels along a 100-bp DNA ladder (FroggaBio, Canada) in Tris-acetate-EDTA buffer and visualized by SYBR Gold stain (Invitrogen). The images were captured using a G:Box gel documentation system (Syngene, UK).
To measure the relative abundance of Vangl1 mRNA, real-time quantitative PCR of Vangl1 and
the reference genes (sequences in Table II.1 – Appendix II), GAPDH, peptidylprolyl isomerase
A (PPIA), and ribosomal protein, large, P0 (RPLP0), was performed using SsoFastTM EvaGreen
supermix (Bio-Rad, USA) in a C1000 Thermal Cycler equipped with a CFX96TM Real-Time
PCR detection system (Bio-Rad). Vangl1 primer pair #1 was used for qPCR. The qPCR data was
then analysed using Bio-Rad CFX Manager (Version 3.0, Bio-Rad). The mean coefficient
variance values of the three reference genes were usually smaller than 0.2 and the M values were
smaller than 0.5, indicating that their expression levels were relatively stable and that they could
be used as reference genes.

**Immunoblotting analysis**

Total cellular protein samples were separated onto 8% SDS-PAGE gel and transferred to
polyvinylidene difluoride (PVDF) membrane. The blot was then blocked with 5% skim milk
solution in TTBS (0.1% Tween-20, 50 mM Tris pH 7.4, 150 mM NaCl) at room temperature for
1 h before probing with primary and secondary antibodies. The primary antibodies used were
rabbit anti-Vangl1 antibody (Sigma, HPA025235, 1:400) and mouse anti-GAPDH monoclonal
antibody (Sigma, G8795, 1:1,000) and the secondary antibodies were horseradish peroxidase
(HRP)-conjugated goat anti-rabbit (Jackson ImmunoResearch, USA, 1:5,000) or horse anti-
mouse (Cell Signalling Technology, USA, 1:10,000). The blot was washed four times of 10 min
after each antibody incubation. Then, the blot was incubated with SuperSignal West Dura (for
Vangl1) or SuperSignal West Pico (for GAPDH) chemiluminescent substrate (Pierce, USA) and
signal was developed in a dark room. The film was then scanned with an Epson Stylus NX115
all-in-one printer.

**Immunofluorescent staining**

Sorted GFP or GFP-Vangl1 cell populations or a mixed cell population containing 80% wild-
type, 10% green fluorescent protein (GFP)-, and 10% GFP-Vangl1 transduced HUVEC were
seeded onto a coverslip or a low-profile 24-well plate (4titude, UK) and, after reaching
confluence, wounded with a P10 tip or left intact, and then fixed with 4% paraformaldehyde
(PFA, Electron Microscopy Sciences, Hatfield, PA, diluted in PBS) at room temperature for 8
min. After being permeabilized with 0.5% Triton-X 100 (Sigma, diluted in PBS) at room
temperature for 5 min, the fixed cells were incubated sequentially with blocking solution [5%
goat serum (Invitrogen) in PBS], then primary antibodies (anti-vinculin (Sigma-Aldrich, V-9131) or anti-Vangl1 (Sigma-Aldrich, HPA025235) at a 1:50 ratio in blocking solution or phallloidin labelled with either rhodamine (Invitrogen, R415) or fluorescein isothiocyanate (FITC) (Sigma-Aldrich, P5282). The cells with primary antibodies were then incubated with blocking solution, and finally tetramethyl rhodamine iso-thiocyanate (TRITC)-conjugated goat anti-rabbit (Sigma-Aldrich, T6778) secondary antibody solution (1:200 in blocking solution) at room temperature. Each incubation lasted for 1 h, with thorough washing with PBS after the incubation with antibody solution. The coverslips were then mounted to a microscope slide using ProLong® Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). Images were taken with a FSM700 confocal microscope (Carl Zeiss Microscopy GmbH, Germany) at a thickness of 2 µm or with an Olympus IX-81 inverted microscope (Olympus Corporation, Japan).

Wound Healing Assay and Wound Healing Tracking

The wells of a 24-well plate (BD) (for the end-point assay) or a low-profile 24-well plate were coated with 314µg/mL PureCol (Advanced Biomatrix, USA) diluted in deionized water with 0.01% HCl for 1 h. After rinsing, HUVEC were then seeded at a density of 84,000 cells/cm². After 24 h, the cells were stained with 500ng/mL of Hoechst 33342 (Invitrogen) and 5µg/mL of wheat germ agglutinin (Invitrogen) or CellMask membrane stain (Invitrogen) in EGM-2 at 37°C and 5%CO2 for 30 minutes. After rinsing with PBS once, the wells were scratched using a P10 tip and then rinsed twice more. Endothelial Serum Free Medium (GIBCO, USA) supplemented with 1% BSA and 2ng/mL bFGF (E-SFM) (Invitrogen, Canada) was added to each well. The plate was transferred to an ImageXpress Micro high-content screening microscope (Molecular Devices, USA) for imaging.

In the end-point assay, the plates were returned to an incubator for 7 h, then fixed with 4% PFA at room temperature for 10 minutes. After rinsing, cells were stained with rhodamine phalloidin. The stained areas at time 0 and 7 h were measured using the morphometry analysis module in MetaXpress software (Molecular Devices), and subtracted from one another to determine the change in denuded area for each well. All wells were then normalized by dividing by the average area change for the corresponding controls, such that wound closure rate is expressed as a percentage of control.
For the tracking assay, the cells were imaged every 30 minutes for 9h. Cell tracking algorithms provided with the MetaXpress software package (Molecular Devices, USA) were used to track nuclear positions over time, and a custom-made program in MATLAB was used to analyse the data.

Cell Tracking Assay

For confluent sheets, HUVEC were seeded at a density of 62,500 cells/cm² in 96-well plates and allowed to attach for 20-24 h before performing cell tracking. 2500 cells/cm² were seeded for sparse cell experiments. Cells were stained with 500ng/mL of Hoechst 33342 (Invitrogen, Canada) in supplemented EBM-2 at 37°C and 5%CO2 for 30 minutes. The dye was washed using PBS, and E-SFM added to each well. An ImageXpress Micro high-content screening microscope was used to take images of the cells every 20 minutes for 8 hours. MetaXpress cell tracking algorithms were used to track cells as above, and MATLAB and R custom-made software were used to analyse the data.

Figure 6.2 - Schematic showing cell density speed correction process: A trendline was created to fit the cell number vs. median speed data for control cells. The equation of that line was then used to calculate a predicted speed for each well using the measured cell number at the first time frame. The ratio between the actual speed and the predicted speed was used as the density corrected speed.
Quantification of cell migration properties

We measured both the total distance travelled by each cell as well as net displacement. These values were divided by total experimental time to obtain cell speed and velocity, respectively. Because the distributions of both cell speed and cell velocity were found to be positively skewed, population medians (from all the cells in the image frame) were chosen as the value to describe central tendency for each individual well. To correct for differences in speed caused by differing cell densities between wells, a linear trendline was generated from wild-type or non-target control cells to describe the relationship between cell number and median speed in the well. This trendline was then used to calculate a predicted cell speed for each well using the measured cell numbers for that particular well. The ratio between the actual and predicted cell speed was used as the density-corrected cell speed (Figure 6.2). This enabled us to detect any differences in speed relative to control cells caused by the treatment alone and not minor differences in cell numbers between wells.

Figure 6.3 - Time-dependent persistence graphs for both Vangl1 and control cells: Persistence is the ratio of net displacement of a cell by the total distance it has travelled. To confirm that persistence measurements were not artifacts of the chosen endpoint, we calculated persistence throughout the experiment and found no differences between GFP and Vangl1 cells at any timepoint.

Persistence was calculated by dividing the net displacement of each cell by its total distance travelled. We used average persistence (calculated at the conclusion of the experiment) after checking that this value changed equally over time for both cell types as recommended previously78 (Figure 6.3).
For the wound healing tracking assay, we defined strips at increasing distances from the edge of the wound (100µm wide for the first slice to account for roughness at the wound front and 50 m for all other). Cells were categorized as belonging to a particular slice depending on their initial position. For quantification, we considered only cells that had migrated more than 2 pixels (6.45µm) between frames. We defined a cell as migrating forward if it had a positive (ie. towards the denuded area) vectorial component of migration orthogonal to the direction of the wound. The distance at which average migration was defined as no longer positive was measured by identifying the slice where the average migration in the positive direction was below 6µm. We also defined a “turning movement” as one characterised by a cell changing its direction of migration by more than 30° in the selected analysis time window.

“Woundless Wound Healing” Assay

Plates were coated with 314µg/mL PureCol as in the wound healing assay above. After 1 h, they were rinsed with deionized water and allowed to air-dry. Polydimethylsiloxane (PDMS) (Sylgard 184 Silicone elastomer kit, Dow Corning Corporation, USA) barriers were prepared by mixing curing agent to base at a 1:10 ratio, curing, and then cutting with razor blades immediately prior to placing on plates. The plates were UV sterilized for 20 minutes before seeding and continuing as above. Barriers were removed with sterile forceps.

Grooved plate preparation

Plates with groove topography were prepared as described in detail elsewhere. Briefly, we used soft photolithography to create the grooved topography, which was then replica-molded using PDMS. The resulting grooves sheets were attached to bottomless 96-well plates using a 1:10 mixture of PDMS:hexane (Sigma-Aldrich) and cured for at least 24 h. The plates were coated with a 10µg/mL stabilized fibronectin solution (Biomedical Technologies, Inc., USA) for 1 h, then rinsed with PBS. Cells were seeded and tracked as above.

Time-lapse live-cell imaging shear stress experiments

Microfluidic channels were fabricated and assembled as described previously. Channels were measured on average 4000 microns in width and 5 cm in length with a height of 335 ± 28 microns. After extensive optimization, we chose the following protocol to avoid cell detachment
Microchannels were pre-coated with 100 µg/ml of fibronectin (Sigma-Aldrich) at room temperature for 1 h before Vangl1-modified or control HUVEC were seeded at a density of 50,000 cells/cm². To aid analysis 10% of seeded cells were pre-stained with Vybrant® CFDA-Se cell tracer (Invitrogen) 2 h before seeding. The fluorescent signal of CFDA was bright enough to eclipse that of GFP-Vangl1 so as to delineate individual stained cells and could last for at least 3 days when cells were seeded at high density to reduce cell division inside the channels. This made the cell segmentation during morphometric measurements much easier. However, it was almost impossible to distinguish the CFDA fluorescent signal from that of GFP in the GFP control lentiviral transduced cells. Therefore, the cell population used as control to GFP-Vangl1 transduced cells were non-transduced HUVEC mixed with 10% of GFP control transduced cells. No effect of CFDA staining on cell re-orientation and no difference between non-transduced cells and GFP control transduced cells were observed based on bright-field images. One day after cells reached confluence, steady unidirectional laminar flow with a calculated shear stress of 20 dynes/cm² on the HUVEC layer was applied in the microchannels using a peristaltic pump and a damping reservoir using a setup described previously. Microchannels were placed in an environment-controlled chamber to maintain the temperature at 37°C and HEPES-buffered L-15 medium (ATCC) was used as circulating medium to avoid the use of CO2. Fluorescent images of FITC channel were acquired at 2-h intervals for 24 h using a 4X objective lens on an inverted Olympus IX-81 fluorescence microscope. There was no apparent phototoxicity observed throughout the experiments.

**Morphometric measurements**

The outlines of CFDA-stained cells of time-lapse live-cell imaging experiment were easily defined by applying the top-hat morphology filter in MetaMorph (Molecular Devices) as only 10% of cells were stained and well separated. However, segmentation of unstained cells within confluent sheets in the end-point shear stress experiments was almost impossible. Therefore, cells were fixed with 4% PFA at room temperature for 10 min immediately after stopping flow. After permeabilization with 0.5% Triton-X 100 at room temperature for 5 min, cells were stained with rhodamine-conjugated phallodin (Invitrogen) to label actin cytoskeleton and with DAPI to label nuclei. Images of stained cells were then taken with a 10X objective lens and the outlines of ~30 random selected cells per image were manually traced based on the phalloidin staining. The
angle and shape factor of selected cells were then measured using the Analyse Particles function in ImageJ.

Statistics

All statistical testing was performed using Origin 8. F-tests were used to test for equal variance. Pooled or non-pooled Student’s t-tests were used to test for significant differences when comparing two independent measures with a Bonferroni correction when applicable. Repeated-measures ANOVA was used to test for significance for the shear flow experiments using a Holm-Bonferroni post-hoc test. For all results, p-values of less than 0.05 were considered significant.
6.6 Results and Discussion

6.6.1 HUVEC express Vangl1, but not Vangl2, and expression levels can be modulated using lentiviral vectors

**Figure 6.4 - Expression and modulation of Vangl1 in HUVEC:** A) RT-PCR analysis of Vangl expression in wild-type (WT) HUVEC: WT HUVEC express Vangl1 but not Vangl2. For Vangl1, two different primer pairs and a non-reverse transcriptase control were used to confirm the observed amplicons were specific to Vangl1 mRNA. For Vangl2, three different primer pairs were chosen and no amplicons were observed in HUVEC. Amplicons from a
control cell type, ARPE-19 appeared at the expected locations for Vangl2, suggesting that the lack of Vangl2 expression observed in HUVEC was not due to improper primer selection. **B)** Immunofluorescent staining of a mixed population of HUVEC with an anti-Vangl1 antibody showed Vangl1 protein located at the cell surface and that exogenous GFP-Vangl1 had a similar subcellular distribution as the endogenous Vangl1. Arrow heads point to HUVEC transduced with GFP-Vangl1 and arrows point to HUVEC transduced with GFP control lentiviral vector. The inclusion of GFP control transduced HUVEC in the staining was to rule out the possibility that the membrane fluorescent signal of GFP-Vangl1 was due to GFP. Staining with an anti-Vangl1 antibody showed similar subcellular distribution of protein in all cells, with increased fluorescence at cell-cell junctions. The GFP signal in GFP-transduced cells (arrows) did not localize to the cell membrane unlike that in the GFP-Vangl1-transduced cells (arrowheads). **C)** Gene expression of Vangl1 in HUVEC modulated by our genetic modifications quantified by PCR. Transduction with a GFP-Vangl1 virus caused a small decrease in the level of endogenous Vangl1 expression while increasing the total expression of the protein. Vangl1-targeting shRNA reduced endogenous Vangl1 expression. **D)** RT-qPCR analysis of Vangl1 knockdown in HUVEC. Relative Vangl1 expression relative to non-target siRNA controls. Vangl1-targeting shRNA reduced Vangl1 mRNA level in HUVEC by at least 70%. Results shown are average values from three independent transductions, and PPIA, GAPDH, and RPLP0 were used as reference genes.

We performed RT-PCR to determine the expression levels of Vangl proteins in human umbilical vein endothelial cells (HUVEC) and found that HUVEC express Vangl1, but not Vangl2 (Figure 6.4A). We confirmed this using multiple primer pairs spanning different exons for both proteins to rule out the possibility that the absence of Vangl2 amplicons from HUVEC cDNA was due to the presence of Vangl2 alternative splicing. Furthermore, to ensure that the absence of Vangl2 was not due to improper primer selection, we used ARPE-19, a retinal pigment epithelial cell line, as a positive control. The presence of bands at the expected locations in ARPE-19 samples indicated that the Vangl2 primers chosen were able to amplify human Vangl2, and therefore, we concluded that HUVEC endogenously express Vangl1 but not Vangl2.

To increase endogenous Vangl1 expression levels in HUVEC, we transduced wild-type (WT) HUVEC with GFP-tagged Vangl1. Immunofluorescence analysis indicated that transducing HUVEC with our exogenous GFP-Vangl1 construct resulted in the expression of GFP-tagged proteins with a similar subcellular distribution to that of endogenous Vangl1, including localization at the cell membrane (Figure 6.4B). Furthermore, immunoblotting confirmed that our GFP-Vangl1 transduction increased the overall level of Vangl1 protein in the cells, although some reduction in endogenous Vangl1 levels was observed in GFP-Vangl1 over-expressing cells (Figure 6.4C). To decrease endogenous Vangl1 expression, we transduced HUVEC with Vangl1-targeting shRNA lentiviral constructs. The lack of Vangl2 expression in HUVEC was advantageous for our knockdown experiments, as it precluded the possibility of functional compensation. PCR analysis indicated that our knockdown construct decreased expression of Vangl1 at both the mRNA (by at least 70%) and protein levels (Figure 6.4C and D).
6.6.2 Vangl1 expression levels influence collective HUVEC migration but not individual cell migration

Figure 6.5 - Vangl1 expression levels affected HUVEC wound closure rate but not the migratory behaviors of single cells or individual cells in confluent sheets: A) Scratch wound closure rate relative to WT cells for over-expressing cells or relative to non-specific siRNA controls for knockdown cells. In a scratch wound assay, Vangl1-overexpressing cells closed the wound faster than GFP controls ($p = 2.49 \times 10^{-11}$, $n = \geq 11$ wells from 3 different experiments), while Vangl1-knockdown cells closed the wound slower than non-target shRNA controls ($p = 2.49 \times 10^{-4}$, $n = \geq 8$ wells from 3 different experiments). B) Average speed of sparsely seeded cells relative to WT or non-specific controls. Vangl1 expression modifications had no effect on the migration speed of sparsely seeded single cells ($p = 0.80$, $n = 3$ (data pooled from multiple wells per experiment for 3 experiments), $p = 0.052$, $n = 3$ (data pooled from multiple wells per experiment for 3 experiments), for overexpression and knockdown cells respectively). C) Migration speed of individual cells migrating within confluent sheets normalized to WT or non-specific shRNA control cells and corrected for any density differences (see Figure 6.2). The speed of individual cells migrating within dense sheets is not significantly different in Vangl1-modified cells ($p = 0.39, 0.886$, $n = \geq 9$ wells in 3 different experiments for overexpression and knockdown cells, respectively). D) Average persistence of HUVEC migrating within confluent sheets. The persistence of Vangl1-modified cells did not differ from that of control cells ($p = 0.84, 0.10$, $n = \geq 9$ wells in each of 3 experiments, for overexpression and knockdown cells respectively). For all graphs, error bars represent 95% confidence intervals (CI).
To investigate the impact of Vangl1 expression levels on collective HUVEC migration, we first performed a standard scratch wound assay in which we assessed the extent of cell wound coverage 7 hours after wounding. Importantly, this assay was performed under serum free conditions, but in the presences of basic fibroblast growth factor (bFGF)\(^1\), to ensure the wound healed by collective motion of the cells as opposed to cell scattering to fill the wound by the movement of individual cells into the free space. In our scratch wound assay, we observed that increasing the expression of Vangl1 in HUVEC resulted in an increase in wound closure. Conversely, decreasing Vangl1 expression lowered wound closure (Figure 6.5A).

To better understand this observed dependence of wound closure on Vangl1 expression level, we first assessed if altering Vangl1 expression simply altered individual cell migration speed or velocity to produce the observed alterations in wound healing. To test this, we used live imaging to track sparsely seeded cells and quantified both cell speed (total displacement/time) and cell velocity (net displacement/time). We did not observe any changes in single cell speed (Figure 6.5B) or velocity (data not shown) in Vangl1 modified cells, suggesting that observed changes in wound closure were not due to alterations in individual cell migration behaviours.

Given the role of the PCP signalling pathway in coordinating cellular behaviour, we reasoned that altering Vangl1 expression levels may affect individual cell migration speed only in the context of collective cell migration, but not during single cell migration as assessed by our sparse cell tracking assay. To evaluate the impact of Vangl1 expression levels on individual cell migration in the context of group cell migration, we tracked the migration of individual cells confined within a confluent sheet, in which cell movement requires collective coordination between neighbouring cells. In this confluent sheet cell tracking assay, we observed that Vangl1 modifications also had no effect on individual cell migration speed (Figure 6.5C), velocity (data not shown) or average persistence (Figure 6.5D). This suggested that the migration of Vangl1 modified cells during cooperative movements are not inherently different from that of control cells, at least in the assays used here to quantify collective migration properties.
6.6.3 Cell polarization cues are not sufficient to observe a Vangl1 migration phenotype in HUVEC

![Figure 6.6 - Morphological cell polarization did not induce changes in the migration of Vangl1 over-expressing cells:](image)

A) Atomic force microscopy (AFM) topographic profile of PDMS microgrooved substrates. Groove depth (peak to trough) was ~600 nm. B) Immunofluorescence image of actin fibres (stained using phalloidin) in HUVEC seeded on PDMS microgrooves. HUVEC were observed to elongate and form stress fibers in the direction of the grooves. The groove direction is marked by the double-headed arrow. C) Visualization of the migration tracks of individual HUVEC cells migrating on PDMS microgrooves. Tracks are colored based on the direction of cell migration for that particular cell. Culture on the grooves resulted in biased cell migration primarily along the direction of the grooves (indicated by the double-headed arrow). D) Characterization of the average speed of individual cells migrating within confluent sheets cultured on grooved substrates for GFP and Vangl1 over-expressing cells. No significant difference was observed (p = 0.16, n = at least 3 wells in 3 separate experiments). Error bars represent 95% CI.

Given the known effects of PCP signalling on polarized behaviour\(^4^4, 8^2-8^4\) and the fact that during wound closure cells elongate and polarize in the direction of the wound, we next considered whether Vangl1 modifications influence cell migration speed only in the context of collective movement in which the cells are elongated and undergoing biased directional migration. To assess this, we characterised the migration properties of elongated cells grown on substrates containing microgrooves (Figure 6.6A), which we and others have previously demonstrated induce cell elongation and polarized migration\(^7^9, 8^5\). We confirmed that confluent sheets of HUVEC seeded on microgrooved substrates showed aligned stress fibers (Figure 6.6B), and
biased migration (Figure 6.6C). Tracking of individual cells within the confluent aligned cell sheet, however, showed that there were no significant differences in individual cell migration speed (Figure 4D, p=0.16) between Vangl1-overexpressing and control cells on grooved substrates, similar to observations on flat substrates (Figure 6.5C and D). This result suggested that Vangl1 overexpression did not impact individual cell migration speed during cooperative motion of elongated cells with a biased movement direction.
Figure 6.7 - Endothelial cell realignment in response to shear stress caused by fluid flow was not affected by Vangl1 modifications: A) HUVEC morphology prior to flow exposure. Approximately 10% of cells were stained with CFDA (green) to allow for observation of cell morphology and changes in whole cell orientation. Prior to exposure to flow, HUVEC within a microchannel were randomly organized and displayed a characteristic
cobblestone morphology, as evidenced by both brightfield (left) and CFDA (right) images. B) Angular measurements (median angle) of whole cell alignment as a function of time after initiation of shear flow for overexpressing (left) and knockdown (right) cells and corresponding controls. No statistical differences (p>0.05) between Vangl1-modified cells and their corresponding control were observed. Error bars are 95% CI. C-E) Quantification of cell migration properties using cell tracking of HUVEC during exposure to flow. When cell nuclei were tracked at different time frames in the reorganization process, at the onset of flow (0-7 hours), when cell rearrangements were most prevalent (7-14h), or when cell alignment had begun to plateau (14-21h), no differences were observed between controls and Vangl1-modified cells in migration angle (C, p=0.61, 0.55, 0.33 for n=3 for each of the 3 timepoints respectively), median cell speed (D, p=0.68, 0.57, 0.84 for n = 3 for each of the 3 timepoints respectively), or persistence (E, p=0.31, 0.48, 0.21, for n = 3 for each of the 3 timepoints respectively). Error bars represent 95% CI.

Figure 6.8 - Angular measurements (median angle) of nuclear alignment as a function of time after initiation of shear flow for overexpressing (right) and knockdown (left) cells and corresponding controls. No statistical differences (p<0.05) between Vangl1-modified cells and their corresponding control were observed.

While grooved substrates induce cell elongation and biased migration along the direction of the grooves, these substrates do not provide a physiologically relevant signal to induce a polarized collective cell movement response, such as occurs in the presence of a wound. In vivo, EC are constantly exposed to shear stress from fluid flow that induces cell alignment. Similarly, in vitro, exposure of ECs to fluid flow produces cellular elongation and alignment. We set out to take advantage of this physiologically induced cell polarization to determine whether EC undergoing cell sheet re-organization and alignment in response to shear flow would exhibit differences in the migration properties of GFP versus Vangl1-modified cells as previously observed in the wound closure assay. Before the onset of flow, EC were randomly oriented (Figure 6.7A). Maximum cell re-organization was observed between 7-14h with a plateau in cell orientation being reached between 14-21h. No differences in the median angle of orientation were observed between Vangl1-modified and control cells at any of the time points assessed (Figure 6.7B and
Figure 6.8), nor was the rate of cell re-orientation different. Furthermore, by tracking the movements of individual cells during the re-organization process, we also did not observe any differences in the migration dynamics of Vangl1 and GFP cells in terms of angle of migration (Figure 6.7C), speed (Figure 6.7D), or persistence (Figure 6.7E) at any of the time point windows (0-7, 7-14, or 14-21h) during the re-orientation process. Together, these results suggested that Vangl1 modifications did not affect the reorganization process despite the presence of polarity and elongation cues from a physiologically relevant signal.

6.6.4 Creation of free space is not sufficient to observe a phenotype in Vangl1 over-expressing HUVEC

**Figure 6.9 - Vangl1 over-expression did not result in increased sheet closure rate in the presence of only free space:** A) Schematic of a “woundless wound healing” assay procedure. Wells were coated with collagen and a PDMS barrier was placed in the well to prevent cell adhesion. Cells were then seeded around the barrier and cultured for 24 hours to ensure junction formation, after which the barrier was removed to generate a region of free space for cell migration. B-C) Fluorescent images of the edge of a “woundless wound” (B) and a wound created by scratching (C). Green – F-actin (stained by phalloidin) and Blue – Nucleus (stained by Hoechst 33342). The cells in the “woundless wound healing” assay aligned to the barrier and formed a smooth edge that was not disturbed by barrier removal. Specifically, the wound edge appeared straighter in the woundless case than that of a scratch wound.
(as observed from the roughness of the edge in the scratch picture), suggestive of minimal cell damage on barrier removal. D) Wound closure rate relative to WT control cells for woundless versus wounding assays. Wound closure rates were higher in GFP-Vangl1 cells compared to GFP cells in scratch wound assays (p=0.019, n = at least 7 replicates) but showed no significant differences (p=0.13, n = at least 7 replicates) in the “woundless wound healing” assay.

Given that Vangl1 modification appeared to not impact cell migration properties in any of the collective assays performed that did not introduce a scratch wound assay into the sheet, we next set out to determine what aspects of a wound are necessary to observe an impact associated with Vangl1 modification. Specifically, we reasoned that introducing a wound into a sheet introduces both cellular damage, which releases chemical signals from damaged cells and free space, which exposes cells at the wound edge to a lack of cell-cell junctions on at least one side of their membrane and reduces restrictions on their movement caused by other cells. To determine if free space alone was sufficient to observe a difference between Vangl1 modified and WT cells, we generated damage-free wounds in cell sheets by removal of a barrier structure present within the cell sheet (Figure 6.9A). This “woundless” wounding assay prevented significant visible cell damage at the wound edge (Figure 6.9B versus C) while enabling the introduction of free space into the cell sheet. In contrast to the standard wound closure assay, when we performed the woundless wounding assay, we did not observe any difference in the wound closure rates of GFP versus Vangl1 over-expressing cells (Figure 6.9D). This suggested that the presence of free space alone was not a sufficient cue to trigger the observed effects of Vangl1 on collective EC migration, but rather that a specific signal associated with damage introduced during wounding is likely critical. We did observe, however, that the cytoskeletal arrangement of cells in the woundless case were aligned parallel to the direction of the wound due to contact guidance produced by the barrier feature (Figure 6.9B) so we cannot rule out that this effect also influenced the lack of phenotype observed in this assay.
6.6.5 During wound closure, Vangl1 modified HUVEC move straighter and are more directed into the wound resulting in increased wound closure rates.

![Figure 6.10 - Actin and vinculin distribution in GFP and Vangl1 overexpressing cells at the wound edge: EC sheets were wounded and allowed to close for between 10 mins and 4h before fixing. The actin cytoskeleton was stained with phalloidin and vinculin was stained with an antibody against vinculin. The wound is located at the top of each image. Vinculin is visible as puncta throughout the cells, examples of which are indicated by arrowheads in the images. Scale bars are 40µm.](image)

Having determined that the introduction of a wound specifically was important for observing an effect of Vangl1 modification on sheet closure rate, we next set out to determine how the behavior of individual cells at the wound edge differed between GFP and Vangl1 cells to produce differences in wound closure rate. To do this, we assessed both the actin cytoskeletal
organization, which is known to undergo a series of characteristic changes after a scratch is introduced\textsuperscript{30}, and vinculin distribution in GFP versus Vangl1 over-expressing cells at the wound edge at different time points after wounding (Figure 6.10). In both GFP and Vangl1 modified cells, actin was observed to align parallel and then perpendicular to the wound edge by 4h after wounding as expected from previous studies\textsuperscript{30}. Vinculin was also visible in the protrusions at the wound edge as expected in both Vangl1 and GFP cell types.

Figure 6.11 - Schematic of the wound healing stripe analysis method: A) The wound was divided into slices 50µm in depth (except for the first slice, which was 100µm in order to accommodate the roughness of the wound edge). Cells were assigned to the slice in which they were located in the first timeframe for the whole experiment. B) Tracks of individual cell movement coloured by direction of movement for all cells in an entire well (left). Whole well data makes it difficult to discern spatial differences in behavior. The slice method (right) allows for a clearer understanding of how different populations of cells are behaving at different locations from the wound edge.
Figure 6.12 - Vangl1 overexpression results in an increased wound closure rate in the wound healing assay because cells move straighter but not faster: A) Average speed (calculated over total time) of the cells as a function of their starting distance from the wound. For each slice, for all the objects in that slice at time 0, the average speed over all time was calculated. Cell migration speed was significantly greater at the wound edge for both GFP and Vangl1 modified cells (p < 0.0043, n = at least 9 replicates) but speed between GFP and Vangl1 cells was not statistically different at any distances (p (after Bonferroni correction for 12 tests) > 0.07, n = at least 7 replicates). B) Average net displacement perpendicular to the wound for cells in strips at different distances from the edge. For each slice, for all objects in that slice at time 0, we calculated the average net displacement along a vector 90° to the wound. Vangl1 cells had a significantly larger component of displacement perpendicular to the wound than GFP cells in slices up to 600 microns from the wound. C) Distance at which point average forward vector displacement becomes <6µm for GFP vs. Vangl1 cells. The distance over which cells show a biased movement into the wound is significantly greater for Vangl1 versus GFP modified cells (p = 0.003, n = at least 9 replicates). D) Percentage of cells whose migration vector included a positive component into the wound (i.e. were directed towards the direction of the wound). Schematic showing definition of forward migration shown in bottom corner of graph. A significantly higher percentage of Vangl1 cells versus GFP cells were observed to move in the direction of the wound in slices 1, 2, 3 and 5 (p < 0.05, n = at least 9 replicates). The percentage of Vangl1 cells moving with a forward migration component was higher than in GFP cells up to 250 microns from the wound location (defined by the first slice in which no statistical difference was observed between Vangl1 and GFP cells). E) Persistence (calculated over 9h) as a function distance from the wound. Vangl1 cells in slice 1 (up to 100 microns from the wound edge) exhibited significantly increased persistence compared to GFP cells (p = 0.001183 for n = at least 9 wells). F) Percentage of cell movements that did not involve a turn (defined here as migrating in a direction that differs by more than 30° from the migration direction in the fourth previous timeframe and shown schematically in bottom corner of the graph) as a function of distance from the wound. A significant difference was observed in the percentage of cell movements not involving a turn between GFP and Vangl1 modified cells in the 2 slices (up to 150 microns) closest to the wound only (p = 0.0011 and 0.0087, n = at least 9 replicates). G) Average angular deviation during a turning event as a function of distance from the wound. Significant differences were observed in the average angle of turning between GFP and Vangl1 modified cells in the first slice (p = 0.0004, n = at least 7 replicates), but none in the remaining layers (p>0.24, n = at least 7 replicates)

Having observed no obvious differences in the cells sheet organization at the wound edge, we performed individual cell tracking during wound closure to quantify the migration properties of individual cells as a function of their distance from the wound. Specifically, we divided the cell sheet into strips sequentially from the wound edge and measured a number of different migration parameters for cells that originated only from within that strip region at the first time point (Figure 6.11). Using this spatially stratified analysis method, we found that cells at the wound edge move slightly faster than cells deep within the cell sheet (Figure 6.12A), but that both GFP and Vangl1-modified cells migrated at similar speeds both at the wound edge and deep within the sheet (Figure 6.12A). When the component of cell migration perpendicular to the wound was quantified as a function of distance from the wound, however (Figure 6.12B), we found that Vangl1 cells had a larger component of movement directed in the direction of the wound compared to control GFP cells, up to ~500 microns from the wound edge. Indeed, the distance over which biased migration into the wound was observed (defined here as the distance at which the average movement perpendicular to the wound becomes zero, indicative of random motion) was significantly greater for Vangl1 versus GFP over-expressing cells (Figure 6.12C). Consistent with this, the number of cells in the sheet moving in the “forward direction (defined here as
within ±90° of the direction of the wound) was increased at the wound edge and was greater for Vangl1 cells versus GFP cells up to 300 microns from the wound edge (Figure 6.12D).

Figure 6.13 - Percentage of cell movements that do not involve a turn from the previous time point as a function of the number of time points elapsed between measurements for cells in the layer closest to the wound edge: Examining the turning behaviour by considering how it differed as the timeframe examined increased allowed us to understand the relatively importance of cell “wiggling” and select an analytical time window appropriate for capturing the relevant cell turning behaviour.

To further explore the migration changes that produced this observed increase in directed motion into the wound in the Vangl1 cells, we assessed if increased directedness resulted from a reduction in cell turning during migration. We first measured average cellular persistence (calculated by displacement/total distance) as a function of distance from the wound edge and observed that Vangl1 modified cells exhibited greater persistence than GFP control cells but only for cells located immediately at the wound edge (Figure 6.12E). The value of persistence results from the combination of how much a cell turns in terms of both frequency and angle. To assess turn frequency, we measured the proportion of cell turning events that occurred during migration by quantifying the percentage of cell movements that did or did not involve a turn as a function of distance from the wound edge (Figure 6.12F). Specifically, we classified a cell to have turned if it changed its direction by more than 30°. We chose to examine this metric using an analytical timeframe of every four time points (two hours) specifically because when we assessed how the percentage of cell movements that do not involve a turn varied as a function of the time window
over which migration direction was compared (Figure 6.13), we found that this metric began to stabilize when calculated over four time points for both Vangl1 and GFP cells. Our analysis indicated that the percentage of cell movements not involving a turn was significantly higher in Vangl1 cells but only at the edge of the wound (up to ~200 microns from the wound) (Figure 6.12F). We also assessed the distribution of turn angles during turning event and found that, in layers right at the wound edge, Vangl1 cells deviated from their previous direction less (turned less sharply) than control cells (Figure 6.12G) suggesting that both turn frequency and turn magnitude are affected by Vangl1 modifications. Together, these data suggested that Vangl1 cells do not migrate faster than control GFP cells but rather that increased wound closure rate results from more directed cell migration of Vangl1 modified cells in the direction of wound due to cells at the wound edge undergoing less frequent turning as well as lower angle turns at the front of the moving sheet.

In this work, we set out to understand the effect of the PCP protein Vangl on EC migration and coordination during cellular re-organization. We found that Vangl1 affects the migration of EC in the scratch wound assay, but not in other single cell or collective migration and cell reorganization assays. Additionally, our results suggest that Vangl1 expression levels impact specifically directional migration in this context and that specific cell damage cues are necessary for the observed phenotype. To our knowledge, this is the first report of the role of a PCP protein specifically in the collective and directed migration of EC. Our data suggest that Vangl1 impacts specifically the frequency and magnitude of cell turning during wound closure resulting in increased movement into the wound. Possible reasons that we speculate could result in this turning behaviour are i) that the cell is more responsive to directional cues from the wound edge or ii) space constraints created by neighbouring cells are more restrictive and limit turning frequency/magnitude. Previous studies link both these possibilities to PCP signalling. For example, in developing myocytes, intercellular PCP signalling was observed to amplify Wnt11 polarity signals resulting in cell alignment, while in WM239A melanoma cells treatment with Wnt5a increased the proportion of cells with polarized CD146 structures induced by the presence of a CXCL12 gradient. It is therefore possible that similarly, Vangl1 expression in our system is amplifying polarity signals that arise from the introduction of a wound. PCP has also been linked to migration constraints that result in directed movement in the context of contact inhibition of locomotion (CIL). For example, in neural crest cells, the PCP proteins Dishevelled, Prickle and
Van Gogh have been shown to impact CIL by preventing the cells from altering their migration direction in response to a cell-cell collision. Hence, it is possible that increased Vangl1 expression could similarly result in increased CIL in EC, such that cells at the wound edge are likely to extend protrusions forward into free space as opposed all other directions where contact would be made with neighbouring cells. This would result in a biasing of their migration toward the wound without affecting cell speed. Furthermore, we would envision this effect diminishing at increasing distances from the wound edge, as observed in our data (Figure 6.12), due to a decrease in the space created by cell movement in the direction of the wound deeper into the cell sheet. A combination of these two mechanisms or others is also possible.

Our observations are also consistent with the molecular mechanisms through which both cell turning and PCP signalling are thought to operate. For example, the opposing polarization of cdc42 and RhoA was recently shown to be responsible for cell turning in response to chemotactic cues. Components of the PCP signalling pathway are also known to lead to RhoA localization at cell-cell contacts during CIL, and to cooperate with the Cdc42/Par6/aPKC complex to promote polarized reorganization of the microtubule cytoskeleton during scratch wound closure. Similarly, coflin is known to affect the turn frequency of migrating cells and has been shown to interact with the PCP pathway. Interestingly, we also observed a lack of effect of Vangl1 modifications in barrier-type wound closure assays. Studies in epithelial cells have shown that the scratch wound assay results in two wave of ERK1/2 activation: one fast and one slow, the former of which is missing in the absence of cell damage. Vangl1 has been identified as a scaffold to a complex that promotes ERK phosphorylation, resulting in increased migration of colorectal cancer cells in a scratch wound assay. Our observations that modifications in Vangl1 differ in wounding versus barrier assays therefore further link Vangl1 and ERK signalling, specifically in situations involving the fast wave of ERK activation. Exploring the impact of these molecular mechanisms and how they relate to signal amplification and CIL in our model are interesting areas for future study.

### 6.7 Conclusions

In this study, we explored the impact of the PCP protein Vangl1 on the single cell and collective migration of EC. We found that Vangl1 affects the migration of EC in the scratch wound assay, but not in other single cell or collective migration and cell reorganization assays. Specifically, we
observed that Vangl1 over-expressing cells do not migrate faster than control GFP cells but rather that increased wound closure rate results from more directed cell migration of Vangl1 modified cells in the direction of wound due to cells at the wound edge undergoing less frequent and shallower turning. These behavioural changes are compatible with previous models of PCP signalling effects on cell migration in which CIL and/or directed migration in response to external polarized signals work in concert to decrease turning and increase the efficiency of wound closure. Overall, our data provide evidence that Vangl1 is involved in coordinating collective behaviours in EC, with a specific role in directed migration.

6.8 Acknowledgments

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6.9 References


Chapter 7
Conclusions and Future Outlook

7.1 Discussion and Conclusions

The work contained in this thesis is an early attempt to acknowledge and understand the guidance of cell migration as a multifactorial problem in which combinations of the components (individual guidance mechanisms such as chemotaxis, cell-cell interactions or topographic features) may have synergistic effects. It also suggests that current knowledge about collective cell migration inform the design of new, more powerful tools to study and describe how guidance cues affect cell migration.

7.1.1 Guidance of Cell Migration Arises from a Complex Signalling Milieu

Figure 7.1 – Non-exhaustive summary of the signals a cell might be exposed to during cell migration: A migrating cell can be exposed to a variety of signals (such as chemical gradients; electrical/magnetic fields; external forces arising from fluid flow or changes in the underlying substrate; topographic cues; and mechanical and chemical interactions with surrounding cells), each of which has been shown independently to affect migratory behaviour. Recent work\(^4\text{-}^6\) has shown that the outcome when cells are exposed to multiple signals at one time is not always not always intuitive, which highlights the importance of examining cell migration events in the presence of guidance cue combinations that reflect the cells’ natural environment.

In their natural environment, cells are exposed to a variety of signals (Figure 7.1) that inform their behaviour\(^1\text{,}^2\). These signals can include chemical signals, such as growth factors and paracrine/endocrine hormones; mechanical signals, including pushing and pulling from their neighbours, forces arising from fluid flow, and stiffness and topographic features from their
substrates; and others such as electric and magnetic fields. In cell migration, these complex signalling environments are further complicated by the dynamic nature of the cell behaviour in question.

Signalling complexity is one of the impetuses that drive the use of in vitro models to understand how signals affect cell behaviour. In vitro models make it easier to perform systematic manipulations of the signalling environment while controlling other variables than in vivo models do. However, those using these models often fail to acknowledge that cells naturally integrate multiple signals, and that this integration may result in synergistic effects which are not obvious when examining the impact of each signal in isolation.

Recent work in the field of collective cell migration has proven that some previously “ignored” cues, such as mechanical cell-cell interactions, can have significant impact on cell behaviours\(^3\). For example, cells seeded at confluent densities respond more strongly to topographic cues than those seeded at subconfluent densities\(^4\); confluent sheets migrate slower, but do not scatter on stochastic topographies, unlike on flat substrates\(^5\). These findings demonstrate the importance of considering any potentially overlooked signals that may be inherent to a particular assay or experimental setup.

Further, that the combination of these guidance mechanisms results in unexpected behaviours underlines the importance of studying the effects of these mechanisms in a combinatorial manner. For example, our finding in Chapter 6 that the Planar Cell Polarity signalling pathway affects directed cell migration, but that this effect depends on damage signals from scratching and not other polarizing cues highlight the importance of examining how the impact of a signal may differ depending on others.

### 7.1.2 The Effect of Combining Guidance Cues on Collective Cell Migration

In this work, we performed systematic analyses of how guidance cues combine to affect collective cell migration. In chapter 4, we focused specifically on the effects of cell-cell interactions and topographic cues, and examined how migration changes when these cues (plithotaxis and topographic cues) are presented separately or together. We also confirmed that combining these cues can result in propagation of topographic guidance to cells not directly exposed to them. In chapter 6, we explored a more complex combination of signals in
endothelial cells, which are a more relevant model due to their *in vivo* behaviour. We explicitly separated the different cues that arise from the scratch wound assay and presented these to cells to determine the impact of individual cues on cell migration. Further, we examined how combinations of cues in the scratch wound assay are resolved in the context of normal versus modified Planar Cell Polarity signalling, a signalling pathway thought to impact how cells coordinate their motion with their neighbours.

While guidance cue combinations have previously been shown to affect both directed behaviour and random migration\(^6\), this work and others demonstrate that directional metrics are more informative than other migration metrics (such as speed) in regards to the effect of guidance cue combinations. Knowing that grooved topography results in bidirectional migration bias along the direction of the grooves informed the decision to measure the deviation in migration angle from that axis in chapter 4. Similarly, in chapter 6, we found that taking into consideration that cells will migrate in a directed manner when exposed to damage cues (as opposed to randomly in the absence of these or other directional cues) allowed us to measure propagation of these cues and how it differed in Vangl1-modified cells versus controls.
7.1.3 The Current Arsenal Used for the Study of Collective Cell Migration Needs to be Expanded

Table 1 – Migration metrics utilized in this thesis: Characterization of migration requires appropriate quantification metrics. A number of the metrics described below have been traditionally been used to describe migration, while those developed in this work are clearly marked so.

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<th>Metric</th>
<th>Behaviour Measured</th>
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<tr>
<td>Inherent individual cell speed (µm/h)</td>
<td>Cell speed is controlled by multiple mechanisms, including actin polymerization rate, strength of cell-substrate adhesion, tail-retraction, etc.</td>
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<td>Total displacement during experiment (µm)</td>
<td>Net displacement characterises cell migration similarly to an endpoint experiment, in which the final location matters more than intermediate steps taken</td>
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<tr>
<td>Efficiency of directed movement</td>
<td>The persistence ratio measures how efficiently a cell moves away from its original position. This value is affected by turning frequency and turning angle, and instantaneous speed changes.</td>
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\[
\text{Total Distance} = \sum X_i \\
\text{Speed} = \frac{\text{Total Distance}}{\text{time}} \\
\text{Net Displacement} = Y \\
\text{Persistence} = \frac{\text{Net Displacement}}{\text{Total Distance}}
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<td><img src="image" alt="Cell track" /></td>
<td>Changes in migration efficiency over time</td>
<td>Proposed by 7 to avoid noise artifacts that may affect persistence measurements in cells with low persistence</td>
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<td>Correlation length (µm) -</td>
<td>Average size of groups that move in a correlated (ie. In the same direction) fashion.</td>
<td>Originaly developed for statistical mechanics, can be used to describe coherent motion. However, it assumes rotation symmetry.</td>
</tr>
<tr>
<td>Average dot product between migration vectors of all cells, described by:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V(r) = \frac{1}{N} \sum_{i=1}^{N} \left( \frac{1}{n} \sum_{j=1}^{n} \frac{\vec{v}_i \cdot \vec{v}_j}{</td>
<td>\vec{v}_i</td>
<td></td>
</tr>
<tr>
<td>$N = \text{All cells, } n = \text{number of objects within radius } r \text{ from } i, \text{ current object of interest. } j \text{ all objects (other than } i) \text{ within radius } r$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metric</td>
<td>Behaviour Measured</td>
<td>Significance</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Stream length (µm)</td>
<td>Average size of groups that move in a correlated (ie. In the same direction) fashion.</td>
<td>New metric proposed in this work (Chapter 3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Describes the distribution in the widths of groups migrating coherently, in order to avoid conflating aligned migration at the front and back of the cell with aligned migration at the sides of the cell.</td>
</tr>
<tr>
<td>Propagation length (µm)</td>
<td>Distance away from a guidance cue at which the migration is no longer influenced by that cue.</td>
<td>New metric proposed in this work (Chapter 4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Measures how far away from a guidance cue (in this case, grooved topography) cells migrate as though they were directly exposed to it.</td>
</tr>
<tr>
<td>Metric</td>
<td>Behaviour Measured</td>
<td>Significance</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------</td>
<td>--------------</td>
</tr>
<tr>
<td><strong>Migration Angle (degrees) and Forward Migration (%)</strong></td>
<td>Direction of migration of each cell in comparison to the direction of the original wound. <strong>New metric proposed in this work (Chapters 5 &amp; 6)</strong></td>
<td>This can be used to calculate how many cells as a percentage of the total population is migrating in the “forward” direction (ie. Into the wound), and compared to random migration, in which half the cells are expected to migrate forward while half migrate backward in order to determine how far away from the wound cells are feeling the effects of the wound.</td>
</tr>
<tr>
<td><strong>Turning vs. Non-turning movements (alpha) and deviation angle (degrees)</strong></td>
<td>Change in migration direction between frames. <strong>New metrics proposed in this work (Chapters 5 &amp; 6)</strong></td>
<td>This metric improves upon the persistence metric by analyzing the frequency of turning during migration. Additionally, the average angle of turning movements can be calculated to determine whether turn magnitude is affected.</td>
</tr>
</tbody>
</table>

The systematic study of collective cell migration is relatively new, and makes excellent use of tools developed in other fields such as fluid dynamics\(^8\), \(^9\), condensed matter physics\(^10\), \(^11\), ecology\(^12\), and chemistry\(^13\), \(^14\). That systems in these fields are also complex and dynamic, and
that many behaviours share similarities makes it reasonable to borrow from existing fields rather than developing brand-new analytical frameworks to analyse collective cell migration.

However, for all the similarities shared between cells and fluids, colloidal systems, migratory animals, and diffusing chemicals, there are also important differences. Cell migration is active and polarized in ways that cannot be captured by the study of inert substances\textsuperscript{10}; cells also lack cognitive abilities that impact the migration of more complex higher organisms\textsuperscript{15}.

In this thesis, we developed a number of new metrics (Table 1) to describe cell migration, and particularly collective behaviours. Some of these metrics, such as propagation distance\textsuperscript{4}, are question-specific and cannot easily be adapted to general cell migration studies. Others, like stream length\textsuperscript{16} described in Chapter 3, which characterises the sizes of groups migrating in a coherent manner; \textit{or} like the $\alpha$ described in Chapter 5, which measures the change in directionality over time\textsuperscript{,} provide meaningful information about cell migration behaviours more generally. Importantly, these metrics are unique in that they were devised specifically for cell migration, and so incorporate information about how cells behave: for example, stream length focuses specifically on cells that are located to the sides of the migrating cell of interest because cells in front and behind a) are likely to be migrating in the same direction, and b) are not being steered by their interactions with the cell of interest\textsuperscript{17}. We also highlight the importance of stratifying/spatially separating the analytical frames during studies of collective cell migration: Given how heterogeneous both cellular behaviour and the environment in which these cells are migration are, failing to separate cells into different domains may result in a significant loss of information.

While we present some cell specific metrics, even these have limitations. For example, stream length is not able to capture what happens when streams moving in different directions meet each other head-on. This may miss potentially interesting dynamics occurring in the axis along which cells migrate. Compared to correlation length, stream length also lacks granularity, because it is calculated by examining the behaviour at specific intervals as opposed to in a continuous fashion. It is worth noting that the values for both correlation length and stream length depend on the selection of an arbitrary cutoff point after which cells are believed to no longer be migrating in a correlated fashion ($\pi/1.7$ in the stream length case, which is approximately the asymptotic value at which the correlation curve plateaus\textsuperscript{16}); the selection of
this cutoff point will affect the final values. Additionally, streams are not static entities, nor are their shapes constant or regular. A dynamic stream participation metric or combination of metrics (which were able to account for the changing number of cells participating in a stream, as well as shape and directionality of the stream compared to its neighbours, while still measuring individual cell behaviour) would perhaps better capture cell coordination by quantifying both the spatial and temporal dynamics of cell-cell cooperation.

7.2 Future Outlook

Collective cell migration is a growing field of research that has the potential to have a significant impact on our understanding of development as well as our strategies for treatment of disease and injury. That the field is in its infancy makes it an ideal time for introducing methodologies of purposeful design in which all potential factors that may impact behaviour are considered during experimental design and data analysis. Purposeful design is also critical in the design of new metrics to characterise collective cell migration.

The work presented here as well as that of others\textsuperscript{18,19} demonstrates that the guidance mechanisms of cell migration can act in a synergistic manner. Incorporating systematic methodologies for dealing with multifactorial inputs, such as factorial design, into the study of collective cell migration is likely to advance the field at a much faster pace than continuing to probe the effects of specific guidance cues in isolation from one another.
Figure 7.2 – Future directions of current work: Two particularly important results were presented in this work in regards to how cells respond to combinations of guidance cues—the finding that topographic cues can propagate as a result exclusively of mechanical cell-cell interactions and the fact that PCP signalling affects endothelial wound closure by altering the turning behaviour of the cells. Understanding whether free space is required for the former, and how PCP signalling is translated into less frequent, shallower turns during migration in the latter will allow for better understanding of how cells prioritize the guidance cues they are exposed to.

While the results in this thesis provide some insight as to how guidance cues are prioritized by cells during migration, mechanistic information is still lacking and needs to be addressed in the future (Figure 7.2). Though PCP signalling was found to affect the directed migration of endothelial cells in the scratch wound assay in a damage-dependent manner, how this PCP signal is relayed to cells remains unclear. Immunofluorescent studies of signalling at the wound edge and farther within the sheet, particularly time courses examining the activation and localization of the Rho GTPases\(^{20}\) and of ERK1/2\(^{21}\), as well as that of other proteins that are part of the PCP signalling pathway, such as Dishevelled, will be able to provide a much clearer picture of how the PCP signalling pathway impacts cell migration. Repeating these experiments in the presence of specific inhibitors, such as ERK inhibitors, inhibitors of microtubule polymerization/depolymerisation, and inhibitors of the Rho GTPases would allow the investigation of the effect of PCP signalling modifications on the dynamics of the wound healing process.

For the work conducted in chapter 4, the question remains of whether spatial constraints arising from the lack of free space may contribute to the propagation of the topographic signals. Examining this propagation in an unconstrained sheet would clarify whether this propagation
continues to occur when cells are not forced to remain in contact with each other. It would also be illuminating to measure force transmission between cells in this assay, perhaps through the use of monolayer stress microscopy\textsuperscript{22}, to better understand how signals are relayed between cells in this system. Along the same lines, confocal live imaging to understand lamellipodial extensions and cell-cell interactions would provide insight as to how these two guidance cues combine to result in biased migration near the groove-flat interface.

Lastly, this work illustrates the importance of recognizing the variety of cues that can affect the migratory behaviour of a cell in any particular situation, and of examining how these cues, both in isolation and in combination, can affect cell migration. It is critical that future research in this field continues to consider the fact that cells rarely encounter guidance cues individually rather than together, and that given the non-intuitive effects that have been observed, more work be conducted to understand the prioritization/integration mechanisms that cells use when undergoing migration.

7.3 References


Figure I.1 - Tracking cells using MetaXpress in a monolayer: A) ARPE-19 nuclei stained with Hoechst 33342 are automatically segmented B), by the software according to local intensity differences, limiting the effects of imaging aberrations. C) The track image produced by the software after tracking the cells in panel A for 30 time frames (see Supplemental Movie S3.1). Scale bars, 200 μm
Figure I.2 - Tracking cells using MetaXpress in a wound healing assay: A) Nuclear image of nuclei (stained with Hoechst 33342) within a wounded ARPE-19 monolayer. B) Corresponding segmentation image for panel A, highlighting the individual nuclei as detected by local intensity differentials. C) Track image produced by MetaXpress after tracking the wound healing for 23 hours (70 time frames) (see Supplemental Movie S3.2). D) Colored tracks produced by our MATLAB track coloring algorithm underscore the coordinated nature of the cells as they move to close the wound. Scale bars, 500 μm.
Figure I.3 - Improved stream identification after track re-coloring: A) Randomly colored tracks of cells grown on nanogrooved substrates that induce aligned motion of cellular streams making stream width difficult to determine. B) MATLAB recolored tracks of cells on grooves shown in panel A, where track color is now defined by the migration direction. The recolored images allow individual streams to be identified and separated much more clearly than the randomly colored images, particularly when the motion of the streams is aligned. Scale bars, 200 μm.
# Appendix II – Supplemental Data for Chapter 6

## Table II.1 – Primers used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pair</th>
<th>Forward/Reverse</th>
<th>Sequence</th>
<th>Target region</th>
<th>Expected PCR product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vangl1</strong> (NM_001172412.1)</td>
<td>1</td>
<td>Forward</td>
<td>AGCCAAGCATATGGCCGGGC</td>
<td>exon 5</td>
<td>190 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CACTGCAACCACCAGCCTTGCC</td>
<td>exon 6/7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Forward</td>
<td>AGCGCAACAGGCAGAATTTGTTC</td>
<td>exon 1/2</td>
<td>698 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>ACACCCGTGGCATGTCAGCCT</td>
<td>exon 4</td>
<td></td>
</tr>
<tr>
<td><strong>Vangl2</strong> (NM_020335.2)</td>
<td>1</td>
<td>Forward</td>
<td>CGGGCTATTCCTCTACAAGTCGG</td>
<td>exon 2</td>
<td>161 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CACTCGTGAGGACGTATGGTC</td>
<td>exon 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Forward</td>
<td>AATGAGTCCACACGAGGGGAT</td>
<td>exon 3</td>
<td>147 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GGAGCAGTCCAGAGGGACA</td>
<td>exon 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Forward</td>
<td>CAGTTGCAGCTGCAGCTGGG</td>
<td>exon 4</td>
<td>565 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>ACTTCTGCATGGCGACGGGCC</td>
<td>exon 6</td>
<td></td>
</tr>
<tr>
<td><strong>GAPDH</strong> (NM_002046.4)</td>
<td></td>
<td>Forward</td>
<td>TCTTTTGCCTCGCCAGCGAG</td>
<td>exon 1/2</td>
<td>93 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TGACCAGGCGCCCAATACGAC</td>
<td>exon 3</td>
<td></td>
</tr>
<tr>
<td><strong>PPIA</strong> (NM_021130.3)</td>
<td></td>
<td>Forward</td>
<td>CGTTGTCTTTCACATGGGCGTGG</td>
<td>exon 1</td>
<td>193 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GGCGTGTGAGATCCACCACCTGGA</td>
<td>exon 3/4</td>
<td></td>
</tr>
<tr>
<td><strong>RPLP0</strong> (NM_001002.3)</td>
<td></td>
<td>Forward</td>
<td>AGGCCTCTCGTGGAAATGGACATC</td>
<td>exon 1/2</td>
<td>190 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCTGACATCTGCTTGGAGGCC</td>
<td>exon 3</td>
<td></td>
</tr>
</tbody>
</table>

Note: the annealing temperature for all PCR reactions were set at 60°C.
Appendix III – Abstracts of Submitted Publications


An algorithm to quantify correlated collective cell migration behavior

Slater B, Londono C, McGuigan AP.

Collective cell migration is an important process that determines cell reorganization in a number of biological events such as development and regeneration. Random cell reorganization within a confluent monolayer is a popular in vitro model system for understanding the mechanisms that underlie coordination between neighboring cells during collective motion. Here we describe a simple automated C++ algorithm to quantify the width of streams of correlated cells moving within monolayers. Our method is efficient and allows analysis of thousands of cells in under a minute; analysis of large data sets is therefore possible without limitations due to computational time, a common analysis bottleneck. Furthermore, our method allows characterization of the variability in correlated stream widths among a cell monolayer. We quantify stream width in the human retinal epithelial cell line ARPE-19 and the fibroblast cell line BJ, and find that for both cell types, stream widths within the monolayer vary in size significantly with a peak width of 40 µm, corresponding to a width of approximately two cells. Our algorithm provides a novel analytical tool to quantify and analyse correlated cell movement in confluent sheets at a population level and to assess factors that impact coordinated collective cell migration.
Nonautonomous contact guidance signalling during collective cell migration


Directed migration of groups of cells is a critical aspect of tissue morphogenesis that ensures proper tissue organization and, consequently, function. Cells moving in groups, unlike single cells, must coordinate their migratory behavior to maintain tissue integrity. During directed migration, cells are guided by a combination of mechanical and chemical cues presented by neighboring cells and the surrounding extracellular matrix. One important class of signals that guide cell migration includes topographic cues. Although the contact guidance response of individual cells to topographic cues has been extensively characterised, little is known about the response of groups of cells to topographic cues, the impact of such cues on cell-cell coordination within groups, and the transmission of nonautonomous contact guidance information between neighboring cells. Here, we explore these phenomena by quantifying the migratory response of confluent monolayers of epithelial and fibroblast cells to contact guidance cues provided by grooved topography. We show that, in both sparse clusters and confluent sheets, individual cells are contact-guided by grooves and show more coordinated behavior on grooved versus flat substrates. Furthermore, we demonstrate both in vitro and in silico that the guidance signal provided by a groove can propagate between neighboring cells in a confluent monolayer, and that the distance over which signal propagation occurs is not significantly influenced by the strength of cell-cell junctions but is an emergent property, similar to cellular streaming, triggered by mechanical exclusion interactions within the collective system.
Multiwell plate tools for controlling cellular alignment with grooved topography

Londono C, Soleas J, Lücker PB, Sathananthan S, Aitchison JS, McGuigan AP.

In many tissues, cells must be aligned for proper function. This alignment can occur at the cellular and/or subcellular (protein/molecular) level. The alignment of cytoskeletal components, in fact, precedes whole cell alignment. A variety of methods exist to manipulate cytoskeletal and whole cell alignment; one of the simplest and most predictable involves seeding adherent cells onto defined substrate topography. We present here two methods to create grooved multiwell plates: one involving microfabrication, which allows for custom design of substrate topography, and a simpler, inexpensive method using commercially available diffraction gratings. We also include methods for manual and automatic quantification of cell alignment.
Compartment boundaries are essential for ensuring proper cell organization during developing embryo and in adult tissues, yet the mechanisms underlying boundary establishment are not completely understood. A number of mechanisms, including (i) differential adhesion, (ii) differential tension, and (iii) cell signalling-mediated cell repulsion, are known to contribute and likely a context-dependent balance of each of these dictates boundary implementation. The ephrin/Eph signalling pathway is known to impact boundary formation in higher animals. In different contexts, ephrin/Eph signalling is known to modulate adhesive properties and migratory behavior of cells. Furthermore it has been proposed that ephrin/Eph signalling may modulate cellular tensile properties, leading to boundary implementation. The relative contribution of adhesion, tension and cell repulsion on ephrin/Eph-mediated boundary implementation however remains unclear. Here, using micropatterning of cells over-expressing either EphB3 or ephrinB1, we dissect the relative contribution of these factors. We show that in this system ephrinB1/EphB3-mediated boundaries are accompanied by modulation of tissue-level architecture and polarization of cell migration. Moreover, we provide the first direct evidence that ephrin/Eph-mediated boundary formation is accompanied by modulation of tensile properties of cells.
Diabetic wound regeneration using peptide-modified hydrogels targeting the epithelium, an alternative to angiogenesis-targeted treatments


There is a clinical need for new, more effective treatments for chronic wounds in diabetic patients. Lack of epithelial cell migration is a hallmark of non-healing wounds. Therefore, targeting re-epithelialization, which mainly involves keratinocytes, may improve therapeutic outcomes of current treatments that mostly focus on angiogenesis as diabetes often involves dysfunctional endothelium. In this study, we present an integrin-binding prosurvival peptide derived from angiopoietin-1, QHREDGS, as a novel therapeutic candidate for diabetic wound treatments by demonstrating its efficacy in promoting human primary keratinocytes attachment, survival, collective migration, and Akt and MAPKp42/44 activation. The QHREDGS peptide, provided as both soluble supplement and immobilized in a substrate, protected keratinocytes against hydrogen peroxide stress in a dose dependent manner. Collective migration of both normal and diabetic human keratinocytes was promoted on chitosan-collagen films immobilized with QHREDGS peptide. The clinical relevance was further demonstrated by assessing the QHREDGS-immobilized chitosan-collagen hydrogel in full-thickness excisional wounds in a db/db diabetic mouse model, which showed accelerated wound closure compared to peptide-free hydrogel and blank wound controls. Furthermore, the accelerated wound closure was primarily due to faster re-epithelialization and increased amount of granulation tissue formation. There were no observable differences in blood vessel density or size within the wound. Together, these findings indicate that QHREDGS is a promising candidate for new wound-healing interventions that enhance re-epithelialization and granulation tissue formation.
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