

**Experimental and Computational Modelling of Collective Cell Migration in
Peripheral Nerve Repair**

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Abstract

A number of biological processes, such as embryonic development, cancer invasion and wound repair, rely on collective cell migration, a process of cells migrate together in sheets, strands or clusters. Peripheral nerves have a remarkable ability to regenerate and re-establish function after injury. Our lab has previously shown that this process depends on the collective migration of Schwann cell cords into the wound, which guide regrowing axons across the injury site to promote re-innervation. Cell-cell contact dependent signalling between Schwann cells and wound fibroblasts plays a critical role in this process by promoting cord formation and directional migration through Eph/ephrin-dependent cell sorting. However, how the interplay of homotypic and heterotypic cell-cell interactions regulates the coherent collective migration of Schwann cells or which molecular are effectors involved is not fully understood. We combine cell and molecular biology experiments with computational simulations to develop a novel computational model of peripheral nerve repair which simulates the interactions between Schwann cells and fibroblasts at the wound site. First, using methods from statistical physics we analyse experimental *in vitro* data to parametrize our model. Then, we use the model to explore the theoretical requirements for an emerging coordination (i.e. formation of collectively migrating Schwann cell cords) in the system in order to better understand the mechanisms by which peripheral nerve repair occurs

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List of Abbreviations

ABM	–	Agent based model
ADH	–	Adhesion
AJ	–	Adherens junction
CA	–	Cellular automata
CCMABM	–	Collective cell migration agent based model
CoA	–	co-attraction
CCM	–	Collective cell migration
CIL	–	Contact inhibition of locomotion
CIP	–	Contact inhibition of proliferation
CNS	–	Central nervous system
CSR	–	Complete spatial randomness
ECM	–	Extracellular matrix
EDF	–	Empirical distribution function
EMT	–	Epithelial–Mesenchymal transition
Fbs	–	Fibroblasts
GFP	–	Green florescent protein
HD	–	High density
kd	–	Knockdown
LCI	–	Live cell imaging
LD	–	Low density

NC	– Neural crest
N-cad	– N-cadherin
PNS	– Peripheral nervous system
RMS	– Root mean square
PPA	– Point pattern analysis
ROI	– Region of interest
Sc	– Schwann cells
SP	– Self propulsion
TAD	– Turning angle distribution
TASD	– Time averaged square displacement

1

Introduction

1.1 Overview

Collective cell migration (CCM) – the coherent movement of multiple cells connected by cell-cell adhesions – is an essential part of numerous biological processes. It plays a key role in development, where the coordinated motion of large groups of cells is necessary for organ and tissue formation. CCM also occurs in adults during wound healing and tissue repair, angiogenesis and cancer metastasis (Weijer 2009; Rørth 2009; Friedl and Gilmour 2009; Friedl et al. 2012; Scarpa and Mayor 2016). Despite its importance, there are many questions which remain unanswered about how this process is regulated.

In part this is due to the breadth of this topic; there are numerous different modes of collective migrations, signalling pathways and guidance mechanisms which are involved (Rørth 2009; Haeger et al. 2015). However, an understanding of the molecular and cellular mechanisms which regulate this process is essential for our understanding of tissue renewal, wound healing and tumour metastasis.

Key hallmarks of collective migration are that groups of cells migrate in the same direction at similar speeds and coordinate their response to the environment. For cells to coordinate motion and stay together, local cell-cell communication is essential. Thus, both the mechanisms of single cell migration and cell-cell signalling are important in collective cell migration. Detailed cell and molecular biological experiments have been used to elucidate the mechanisms of single-cell migration – which are now well understood (Lauffenburger and Horwitz 1996; Ridley et al. 2003). However, in collective cell migration, cellular contacts alter the distribution of the classical features found in individual migrating cells, leading to the emergence of complex group features.

Computation modelling is a method which is now often used in conjunction with biological experiments to aid in the understanding of complex phenomenon (Benigni and Giuliani 1994; Mogilner, Wollman, and Marshall 2006; Vallabhajosyula and Raval 2010; Gunawardena 2014). Theoretical modelling approaches have also provided important insights into this intracellular actin dynamics (Novak2008 ; Mogilner and Edelstein-Keshet 2002),the impact of the extracellular matrix (ECM) (Hillen 2006; Painter 2009; Schlüter, Ramis-Conde, and Chaplain 2012) and whole-cell motility dependence on multiple factors, (Tozluoglu2013 ; DiMilla, Barbee, and Lauffenburger 1991; Zaman et al. 2005). Due to the complexity of collective cell migration, quantitative biological experiments combined with a computational modelling is suitable to understand better how complex behaviour can emerge from lower order interactions.

CCM plays an essential role in peripheral nerve repair (see section 1.5). From previous work in this system (Parrinello 2010), it is known that signalling between fibroblasts and Schwann cells is necessary for successful repair. I used this as a model system to investigate how directional collective migration can emerge from heterotypic cell-cell interactions between Schwann cells and fibroblasts. I built an agent-based model, in which the behaviours of agents were based on features extracted from *in vitro* cell and molecular biology experiments, and parameterized the model using a quantitative metric of collective cell migration from results from *in vitro* live cell imaging experiments. I then used the model to investigate how cord like collective migration can arise from local changes in Schwann cells behaviour mediated by Schwann cell-fibroblast interactions.

1.2 Aims

The goal of this project was to understand how directional collective migration can arise from direct cell-cell signalling between Schwann cells and fibroblasts. The aims were to:

1. Quantitatively understand how fibroblasts change Schwann cell behaviour.
2. Quantitatively understand how these changes impact the mode of Schwann cell migration.
3. Further the understanding of the role ephrinB2/ephB2 signalling plays in Schwann cell/fibroblast interactions.
4. Build an agent based computational model to replicate the emergence of collective migration in *in vitro* experiments of Schwann cell migration.

5. Use this model to test and explore the understanding developed of how emergent collective migration occurs - i.e. determine which features are necessary.

To do this I combined biological and computational experiments from which I identified key features of Schwann cell/fibroblast interaction, extracted quantitative measures of collective migration, and built a bottom-up mechanistic cell-cell interaction model of collective cell migration. I then parametrised and calibrate the model using quantitative readout of biological *in vitro* data and the used the model to explore what features are necessary to recreate the cord like collective cell migration we observe.

Specifically is this system I look at how signalling between fibroblast and Schwann cells results in directional collective cell migration of the Schwann cells. From a mechanistic perspective I explore how ephrinB2/ephB2 signalling coordinate migration at the cellular level by characterise collective motion in a principled manner. Given the complexity of collective cell migration, I have used an integrative biological and computational approach. Using these approached I investigate how simple interactions between cells can combine to give rise to these emergent phenomenon of collective migration.

Incorporating the measured data into a agent based computation model, I showed that strengthening intercellular adhesion is necessary for generating collective cell movement, however we also showed that polarity and CIL are also necessary. The model thus suggests that not only cell adhesion but also cell motility are require for the emergence of collective cell movement.

1.3 Cell Migration

Cell migration is a broad term used to describe the processes which involve the movement of cells from one location to another. It is an essential process in multicellular organisms, as in an integral part of development, tissue maintenance, wound healing, and immune responses (Raftopoulou and Hall 2004; Krause and Gautreau 2014; Mayor and Etienne-Manneville 2016). Although it is a highly complex process, involving many different signalling pathways and molecules, the molecular mechanisms that control single-cell migration have been studied extensively and are relatively well understood (Lauffenburger and Horwitz 1996; Ridley et al. 2003). To migrate, cells must adopt a spatial asymmetry which allows them to turn intra-cellularly generated forces into motion. The spatial asymmetry reflects the spatial division of the signalling cascades which regulate the different stages of the migration cycle. Cell migration is often described as a multi-step cycle (Sheetz et al. 1999); (1) in response to signalling cues front-to-back polarisation of the cell occurs, (2) this is followed by the formation of protrusions at the leading edge via rapid actin polymerization, (3) these are stabilised via attachment to the extracellular matrix (ECM) by integrin-mediated adhesion complexes, which link the ECM to the actin cytoskeleton and act as signal transduction centres which control cell migration. Then the actin cytoskeleton can generate the forces required to translocate the cell forward. Finally, adhesions at the rear of the cell are disassembled so that the cell can move forward. The activity of the actin-based protrusion and contraction machinery and integrin-mediated adhesions need to be continuously controlled to ensure proper execution of each of the steps described above.

1. Establishing Front-To-Back Polarity

Single-cell migration is fundamentally based on the establishment of a front-to-rear

polarity axis. Polarised cells have regions which are morphologically or functionally distinct from the rest of the cell, and a manifestation of this asymmetry is often a polarized morphology, where a clear distinction between the cell front and rear is observed (Nabi 1999; Lauffenburger and Horwitz 1996). Front-To-Back Polarity is initiated and regulated by the differential activity of small GTPases (Cdc42, Rac and RhoA), which regulate actin dynamics, adhesions and the formation of protrusions. Cell polarity is maintained and often arises in response to environmental cues. These cues can be chemotactic, hapto-tactic, mechano-tactic, electro-tactic, and duro-tactic, or combinations of any of these. They induce polarisation by the spatially regulated recruitment of signalling complexes, which in turn modulate the actin cytoskeleton and mediate the extension of membrane protrusions at the leading edge.

2. Protrusion Formation

Once the cell is polarised rapid actin polymerization at the front of the cell drives the extension of membrane protrusions; lamellipodia (broad and flat protrusions) and filopodia (thin, finger-like protrusions comprised of polymerised actin filaments that are arranged into long parallel bundles) (Varghese et al. 2012). These two types of protrusion are believed to play different roles: the filopodia act as mechanosensory, exploratory tools, while the lamellipodia provide large surfaces that generate traction for forward movement (Xue, Janzen, and Knecht 2010). Typically, lamellipodia and filopodia coexist at the protruding region of migrating cells. However, the number of filopodia are highly variable depending on cell type and environmental conditions. Actin dynamics and organisation are regulated by, vasodilator-stimulated phosphoprotein (VASP), Wiskott–Aldrich syndrome protein (WASP), profilin and the Arp 2/3 complex, which is found localised close to the periphery of protruding lamellipodia. Rac and its effector, p21-activated kinase (PAK) which regulate actin polymerization and protrusion formation are also found at or very near the leading edge (Bravo-

Cordero et al. 2013; Bravo-Cordero, Hodgson, and Condeelis 2014).

Stabilisation

At the forefront of the lamellipodium, integrin-mediated adhesions form, connecting the actin cytoskeleton of the cell to the extracellular matrix to anchor the protrusion and stabilise the membrane protrusions (Sheetz et al. 1999). The establishment of adhesions with the substratum provides traction for the movement of the cell body and acts as a signalling hub for actin polymerization regulation. Integrin-based adhesions mature into focal adhesions which provide the anchor points for stabilising longitudinal actomyosin cables and are maintained until they are at the back of the cell. Due to the dynamic nature of cell motility, integrin-containing adhesion complexes are necessarily also dynamic formations which undergo cycles of formation and disassembly (Webb, Parsons, and Horwitz 2002). Thus, adhesion at the front of the cell at the base of a membrane protrusion turn-over as new adhesions begin to form at the leading edge.

Retraction

To move forward, the cell must detach from the ECM and retracts its trailing edge (Sheetz et al. 1999). The detaching process occurs via adhesion disassembly and RHO mediated actomyosin contractility. Several mechanisms participate in promoting adhesion disassembly; these include microtubule-dependent targeting of dynamin and subsequent endocytosis of some adhesion components; myosin-mediated contractility contributes via regulation of MLC phosphorylation by RhoA and Rac acting through Rho kinase and PAK. Dissolution of adhesive contacts at the rear of the cell is also realised by affinity down regulation through phosphorylation by phosphatases such as calcineurin, or by proteolysis of molecules such as talin by proteases, for example, calpain. Evidence shows that the integrins remain connected to the substrate while the intracellular adhesive components, slide toward the cell body due to the tensile force acting on them and then disperse (Lauffenburger and Horwitz 1996; Ingber 2003). The cytoskeletal components, (e.g. paxillin and α -actin, are not been observed in "footprints" left behind the cell which suggests that cleavage of the cytoskeletal linkage occurs at or very close to integrin.

Force Generation and Adhesions

Adhesion to the ECM/substratum allows the cell to generate traction forces which allow them to move (Sheetz et al. 1999). These adhesions occur mainly via integrin receptors which are a large superfamily of heterodimeric receptors which bind to different extracellular matrix ligands or their partner ligands on other cells. Integrins link the substratum to the actin cytoskeleton and providing traction for migration, and their ligation triggers signalling pathways that regulate protrusion. Adhesion sites are usually spatially restricted and vary from small and dot-like (nascent adhesions or focal complexes) to large and elongated (focal adhesions). The shape, size and functionality of adhesions depend on their localisation within the cell and the cell type.

Those found closer to the leading edge, (within the lamellipodium), or those found in fast moving amoeboid cells are usually smaller, assemble and disassemble quickly and actively promote actin polymerization. Those located away from the leading edge and in mesenchymal cells tend to be larger, more stable and anchor large actin filament bundles. The Rho family of small GTPases have a fundamental role in the control cell movement via the regulation of actin polymerization, actomyosin contractility, actin organisation and microtubule dynamics. In migrating cells, Rac is needed for the formation of new adhesions at the cell front, while Rho is required for maturation of already formed adhesions (Raftopoulou and Hall 2004). It is believed that Rac regulates adhesion turnover either directly through downstream effectors and indirectly by antagonising Rho. Rho plays a role in disassembly of adhesions at the rear of cells. Rho together with its effector, Rho kinase, are thought to promote retraction by altering the affinity and avidity of integrins. The mechanisms by which adhesions assembly occurs are not well understood. However, current evidence favours a sequential recruitment of individual, or classes of, adhesion elements around a nucleation centre rather than the stabilisation of large, pre-formed cytoskeletal complexes.

Guidance cues and Directionality

There are many types of extracellular cues which can influence cell motion. They assume various forms, including soluble and cell-bound ligands, insoluble matrix substrates, electric fields (Rajnicek, Foubister, and McCaig 2007), topographical cues and variations in substrate stiffness. To give rise to directional motion guidance cues must be presented to the cell in an inhomogeneous manner. Direction mechanisms coupled to the underlying motility machinery of cells respond to the asymmetric environmental factor, resulting in directional migration. At the cell level, guidance cues cause attraction or repulsion of protrusions. The frequency and direction of local protrusions which are formed parallel to the front–rear axis of movement is important

in determining directionality (Arriemerlou and Meyer 2005; Andrew and Insall 2007). At the molecular level, the cell integrates information from external guidance cues in its local environment which influence the molecular mechanisms that drive each step of the motility cycle.

Migrating cells can move randomly or in a directional manner. The underlying mechanisms of random cell motion are well-characterised (Vicente-Manzanares, Webb, and Horwitz 2005); however, there is no complete model which explains the complex regulation of directional migration. As described above, cells move by the formation of membrane protrusion. Over time, the position of the dominant protrusion changes, resulting in the cell changing direction and thus exhibiting (over a longer time scale) a random pattern of motion. Cells can overcome their random intrinsic motion and acquire directional migration by maintaining the axis of polarisation in one direction via the formation and stabilisation of actin-rich protrusions or lamellipodia (Lacayo et al. 2007). During directional cell migration (as opposed to random migration), cells bias their direction of motion by coupling their natural motion with guidance from an external cue. Directional movement enables cells to move efficiently between points, whereas random migration allows cells to explore their local environments (Arriemerlou and Meyer 2005; Andrew and Insall 2007; Iden and Collard 2008).

Modes of Migration

The two main types are collective migration and single cell migration. Single cell migration can be further classified as amoeboid- or mesenchymal types of movement (Friedl and Gilmour 2009; Trepap, Chen, and Jacobson 2012). Amoeboid movement is identified by absent or weak adhesions. In blebby amoeboid migration, cells move using a propulsive bleb without using focal adhesions, and cell attachment and are typically rounded, ellipsoid bodies. It is found for example in embryonic cells, leukocytes migrating through the ECM and in some cancerous cells. Another form of amoeboid

migration is characterised by outgrowth of actin-rich filopodia and weak substrate interaction, found, for example, in migrating neutrophils and dendritic cells or tumour cells. Mesenchymal migration involves strong focal attachment to the ECM, cytoskeletal contractility and an elongated cell morphology (Friedl and Wolf 2010). Fibroblasts, sarcoma cells and tumour cells, which have undergone epithelial to mesenchymal transition (EMT), are known to use this mode of motility. Collective cell migration (CCM) is recognised by the cohesive, coherent movement of groups of cells. In the next section, I discuss CCM in more detail.

1.4 Collective Cell Migration

Collective cell migration (CCM) is broadly defined as a group of cells migrating coherently towards a common destination while influencing the behaviour of one another via a stable or transient cell–cell connection (Weijer 2009; Rørth 2009). This process is essential for morphogenetic movements, tissue repair and tumour spreading. Coordinated cohesive migration of cells improves the migratory capacities which individual cell to induce a movement that is faster and more directional.

Cells have been observed to migrate collectively in different modes depending on cell type and the environment (Rørth 2009); in cohesive sheets, cords, branches, clusters, ducts, glands or loose streams. Different modes of collective migration serve different purposes, for example, 2D monolayers of cells migrate intact across the basement membrane during wound healing. In other cases, sprouting ducts and glands often include distinct cell types which migrate together and form a ductal tree or network. The mode of migration can depend on several factors, including adhesion strength between cells, the type of substrate, external migratory signals and cues, mechanical stiffness and dimensionality (Rørth 2009). The term collective migration applies to different instances of cohesive cell movement, which are all variations of the same fundamental process.

Broadly speaking, CCM can be divided into two categories, cohesive collective motion and loose collective motion (see Figure 1.1). In the case of cohesive groups, direct cell–cell contacts maintain the physical integrity of the group and also contribute to the coordination of the motile behaviour of neighbouring cells (Friedl and Gilmour 2009). However, in loose-stream migration (cranial neural crest cells) only transient contacts are maintained (Carmona-Fontaine et al. 2008; Carmona-Fontaine et al. 2011; Woods et al. 2014). Nevertheless, collective behaviour is observed in these loosely bound

streams of cells, indicating that communication either via diffusible cues or by the local modifications of the extracellular matrix (ECM) can also promote cell coordination (Weijer 2009; Rørth 2009).

Collective migration of cells is distinguished by a coordinated movement which is generated by local cell-cell interactions and interactions between individual cells and their environment. Such self-organization is familiar from the collective behaviour of groups of animals, for example shoaling in fish. However, the interactions of cells are restricted to a more limited variety. For a group of cells to move from one place to some form of directional guidance cue is necessary. These signals come in a variety of forms, such as chemical, mechanical or electrical. The molecular mechanisms that control single-cell polarisation and migration can also be applied to collective migration. However, the effect of cohesive bonds between cells and other population driven effects must also be considered (Rørth 2009). Cell-cell adhesions lead to emergent features of the whole group which can, in turn, affect how guidance cues are perceived by the cells. The migration pattern of collectively migrating cells is different from the migration characteristics of the individual constituent cells, and it is characterized by the type and strength of their mutual interaction.

Often the analysis of cell behaviours identifies two types of cell or two cell states. These cell states are typically classified as the "leader cells" and "follower" cells (Rørth 2012; Chapnick and Liu 2014). Leader cells (cells found at the front of the group) integrate signals from the local environment and direct the direction and speed of migration of the entire cell cluster. Follower cells are those cells which are not at the front of the group. The characteristics which make leader cells distinct from follower cells are varied and depend on the system being analysed. Leader cells can be created by exposure to various growth factors. As a result, they appear and behave differently from follower cells. Leader cells usually have many filopodia and can respond to ex-

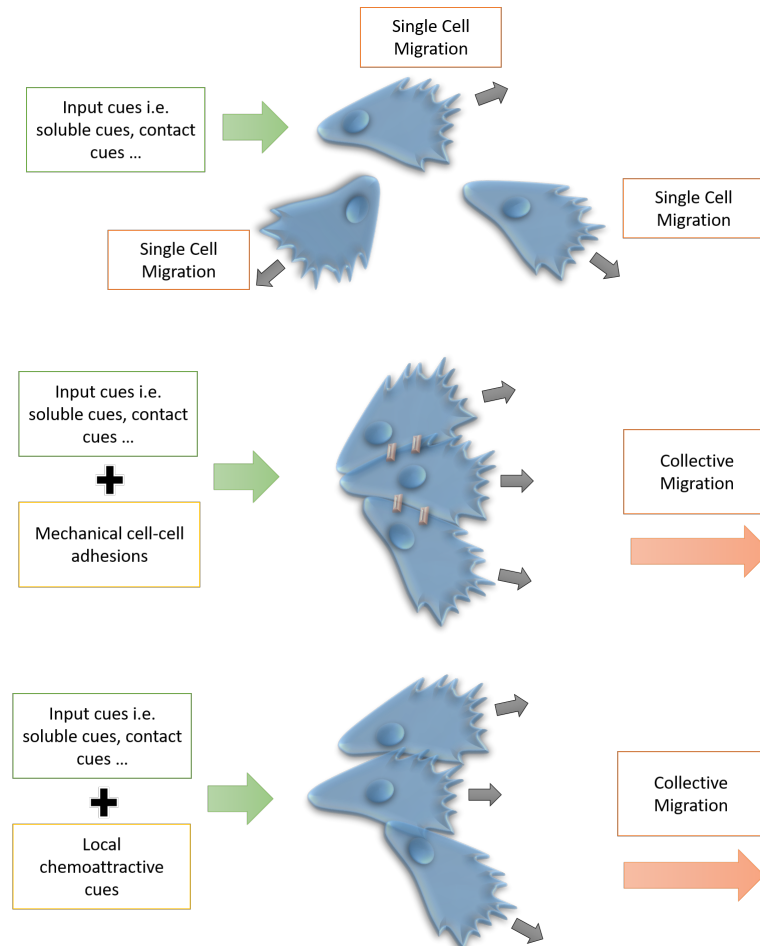


Figure 1.1: Cells can migrate individually (top), as cohesive groups (middle) where adhesion junctions maintain physical connections between cells, or as loose groups (bottom), where local chemical attractive cues and contact inhibition maintain the group.

ternal guidance cues (Ruhrberg et al. 2002; Gerhardt et al. 2003; Ghabrial and Krasnow 2006). As a result of their position in space, leader cells are exposed to higher levels of guidance signals such as chemoattractants and play a major part in ECM remodelling during migration. Behind leader cells, cell–cell contacts impair the formation of a classical leading edge, indicating that the mechanisms driving the migration of so-called followers must be different from those affecting the leader cells. The followers must, therefore, rely on strong cellular connections to polarise collectively. In a migrating single cell, signalling at the rear can modulate the speed and the direction of migration. Furthermore, follower cells can also affect the behaviour of the leaders to modulate the collective movement. In collectively migrating and invading cancer cells; leader cells also express different genes compared with internal and follower cells. The leader cells in collective migration are functionally distinct from the follower cells. However, observing collective migration via live cell imaging shows that in many cases leaders and followers are found to swap places and change roles. Leader and follower cells can therefore be thought of as different cell states, although not different cell types. .

1.4.1 Guidance in Collective Migration

Morphogen Gradients

Gradients of chemicals are known as morphogens are thought to aid in directing growth, cell migration and differentiation. These are typically established, and often play a key role in directing cell migration during development (Rogers K.W 2011; Müller et al. 2013). Alternatively, guidance can occur via dynamic interpretation and generation of gradient signals during migration. In the absence of such pre-established chemical gradients, cells can establish a self-generated chemokine gradient by three actions. They can release a motility-enhancing factors near the front, which creates a positive feedback loop in leader cells, release a chemokine-degrading enzymes which

diffuse down the group of cells and clear chemokines, or express migration-inducing receptors in the leading cell group that bind, internalize, and thus remove chemokines so limiting their activity in follower and trailing cells (Sebastian J Streichan and Hufnagel 2011). This self-generation of a chemotactic gradient induces supracellular polarity, which (for example) is required for directional migration of the zebrafish lateral line.

Proliferation

Instead of directed migration, tissue expansion can also be driven via proliferative increase, in which cell fronts advance from an increased division of cells at the leading edge of the cell population, filling the space adjacent to the population via cell division rather than directed motion (Simpson et al. 2007). Together with spatial confinement, frontal expansion can provide a direction and thus facilitate invasion. One example of proliferation-driven colonisation is found in the mouse gut, where enteric neural crest cells at the migration front have higher proliferation rates than the rest of the population. This facilitates directed migration in the absence of long-range directional cues.

Contact Guidance Cues

Groups of cells interact with the extracellular matrix (ECM) and molecules bound by the ECM. The interaction between cells and the extra cellular matrix (ECM) results in modulation of adhesions and the actin cytoskeleton. However, cells also detect, respond to, and follow the topography of their environment. This is known as contact guidance (Tamiello et al. 2016). If the physical and molecular cues are presented in an inhomogeneous manner they act as a gradient, which results in intracellular signal polarity and movement along the gradient. Group cell migration can be guided by topographic guidance signals and this guidance information can be transmitted between cells within the group. There is evidence which supports the idea that in this

mechanism signal transmission arises from a volume exclusion-type process and is an emergent property that can arise in dense cell populations (Londono et al. 2014).

Cell Signalling Cues

Cell guidance away from an encountered structure is known as repulsion. Haptokinetic repulsion can be mediated through direct cell–cell communication, for instance by the interaction of Eph receptor tyrosine kinase with membrane-bound associated ephrin ligands on neighbouring cells, or through downstream signalling events of other cell–cell adhesion molecules, including N-cadherin (Park and Lee 2015). Repulsive downstream signalling from Eph/ephrins signalling leads to Rac inhibition, disassembly of focal adhesions, and collapse of cell protrusions followed by the cell reorientation itself in the opposite direction (Wang et al. 2010). Furthermore, repulsion results from Rho-dependent contractility at cell boundaries, which prevents stable cell junctions and supports cell and tissue separation (Wang, Chen, and Anderson 1998).

Another guidance mechanism is the combination of contact inhibition of locomotion (CIL) and local co-attraction (CoA), where group cohesion is controlled by the balance of repulsion and attraction between cells (Woods et al. 2014). CIL increases dispersal, while CoA stabilises the dispersion which would otherwise arise from the individual action of CIL. This mechanism can lead to group cohesion and alignment; however, further cues or mechanisms are necessary to induce directionality in the overall migration, for example, confinement via physical boundaries or chemoattractant gradients. This mechanism has been suggested as a general mechanism for collective neural crest cell migration.

It is important to differentiate between the effects of contact inhibition and cell volume exclusion. Cells occupy physical space. Therefore when they come into contact, they

must either steer around, climb over or repel away from each other. CIL is a particular type of repulsive interactions wherein the direction of movement - following cell-cell interaction - is steered away from the point of contact with another cell., i.e., the direction of motion away from the point of interaction (see Figure 1.2 top row). Volume exclusion, however, prevents cells from encroaching on the space occupied by the other cell but permits the cells to move into any nearby space that is empty of other cells, i.e., there is no bias in the direction of movement away from the point of contact (see Figure 1.2 bottom row).

It can be difficult to distinguish a CIL-like mechanism from a purely physical phenomenon of volume exclusion. However, the importance of volume exclusion in cell migration has been studied using continuum models of moving cell populations. The difference in the outcome of interactions resulting from repulsive CIL and directionally unbiased volume exclusion has also been studied in simulations of cell dispersal, where volume exclusion fails to produce the periodic patterning that results from dispersal through repulsive CIL.

Cell-Cell Adhesion and Classical Cadherins

Adherens junctions (AJ)

Adherens Junctions are protein complexes that occur at cell–cell junctions in epithelial and endothelial tissues and enable inter-cellular interaction (Harris and Tepass 2010). They play an important role by maintaining physical interactions between cells, counteracting the forces exerted between cells and by serving as a signalling hub for cell polarity and directed migration. Cell–cell adhesion is mediated by the adherens junction proteins, which include; cadherins, other immunoglobulin superfamily members and integrins, all of which directly or indirectly connect to the actin and intermediate filament cytoskeleton and thereby provide mechanically robust but dynamic coupling.

Classical cadherins; E-cadherin, VE-cadherin and N-cadherin, are the main transmembrane proteins of AJs (Angst, Marcozzi, and Magee 2001).

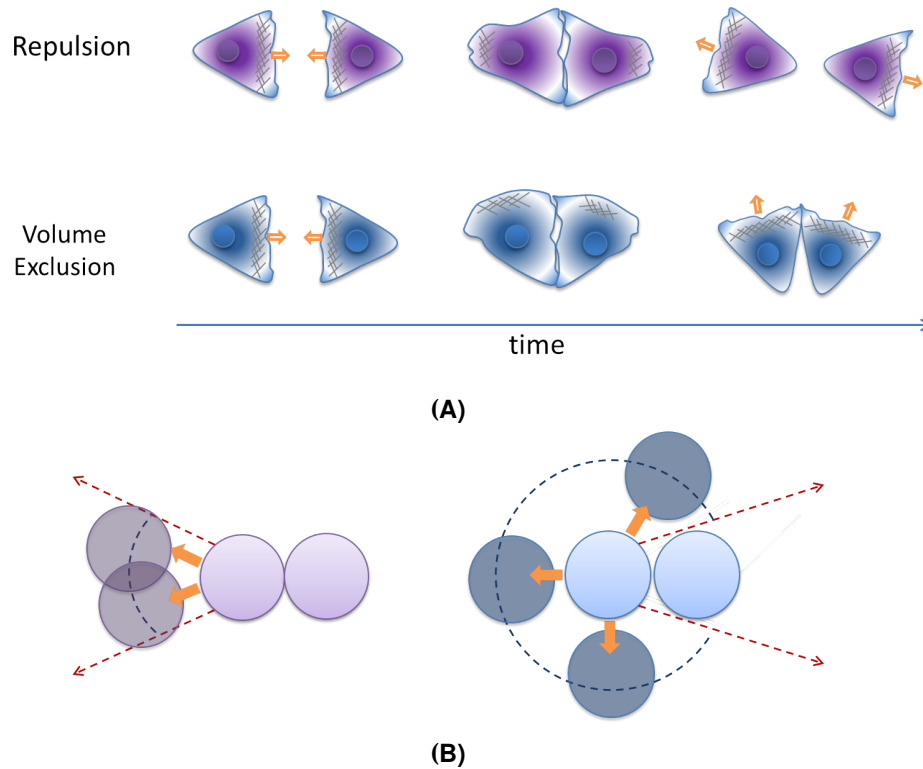


Figure 1.2: CIL vs. volume exclusion Schematic: Schematic of cell–cell interactions. (A) Cells move towards each other (left) and upon interaction (middle) either undergo CIL (top) or volume exclusion (bottom). The type of interaction affects the direction in which the cells can move apart from each other (right). Repulsive interactions - contact inhibition of locomotion- (top, purple), cell signalling results in repolarisation via actin activity (grey crosses) away from the point of contact. This results in cells moving directly away from the point of contact cell. Volume exclusion (bottom, blue), cells block each other’s movement, but can move to any space not occupied by another cell. (B) The space into which cells can move in either case is shown as the dark dashed line between two red lines (second cell shown as stationary for simplicity). (Schumacher et al. 2016)

Cadherins

There are thought to be over 100 different types of cadherins in vertebrates, which are divided into classical, desmosomal, protocadherins and unconventional cadherins. E-cadherin and N-cadherin are both classical cadherins typically found in epithelial and mesenchymal cells respectively and share a common modular structure (Shapiro and Weis 2009). Classical cadherins are composed of a small cytoplasmic component, a transmembrane component and an extracellular segment with five repeated similar subdomains called cadherin repeats, each of which is made of about 110 amino acids which exhibit a folded topology and contain calcium-binding sequences. Calcium ions interact with these sequences to controls the conformation of the extracellular domain, switching its adhesive function off and on. On association with calcium, the extracellular domain of cadherins on a cell undergoes homophilic cis- and trans-interactions with that of cadherins present on the neighbouring cells, forming dense protein clusters that constitute the core of AJs (Meng and Takeichi 2009; Harris and Tepass 2010). The cytoplasmic domains are highly conserved in the classical cadherins. This portion interacts with cytosolic partners, such as β -catenin, α -catenin and p120-catenin. The juxtamembrane part of the cytoplasmic domain is associated with p120-catenin, while the carboxy-terminal half of the cytoplasmic domain binds β -catenin. The catenins associate with various other molecules, including cytoskeletal proteins and their associated regulators. Via these catenins, cadherins are connected to the cytoskeleton and various signalling pathways; consequently, AJs are involved in mechanosensing and mechanotransduction, which contribute to the coordination required for collective migration (Duc et al. 2010; Ladoux et al. 2010).

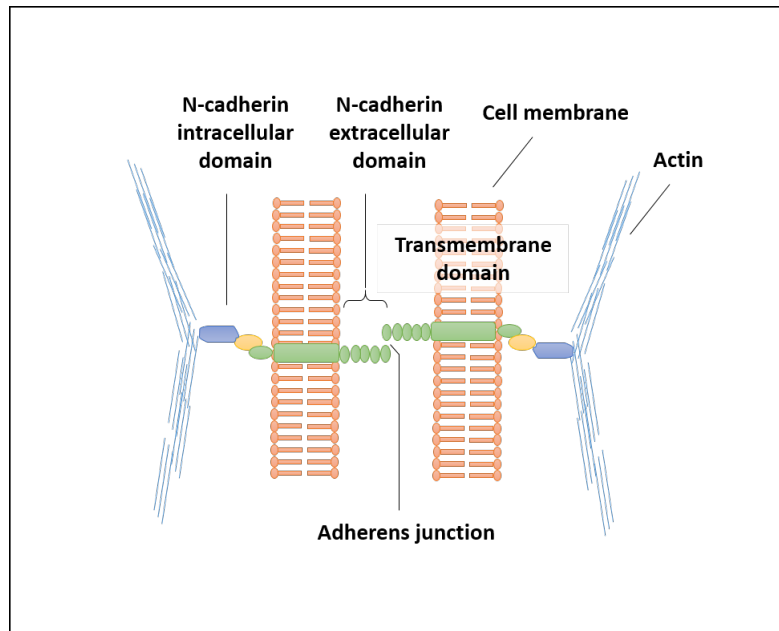


Figure 1.3: Cadherin schematic

AJ Formation and Regulation

In epithelial cells, new cell–cell adhesions are formed in 3 steps: (1) initiation, (2) expansion and (3) stabilization (Theveneau and Mayor 2012). In brief, during the initiation phase, cells explore their local environment using membrane protrusions, which allows them to encounter nearby cells randomly. When the membranes of two cells touch, cadherins present on their surface form homophilic bonds, and this brief contact causes a brief, transient increase in Rac1 activity which is followed by a subsequent increase in RhoA activity. Lamellipodia formation at these sites is inhibited, and instead, the protrusions form sideways, away from the point of contact. As the membrane protrusions extend sideways, they cause further overlap between the cells which promotes new adhesions to form. At the contact site, branched actin is assembled into bundles of actomyosin which are orientated parallel to the cell cortex. As the contact region grows, membrane and actin activity decrease, allowing a stable connection between the adherens junction and actin cytoskeleton to be maintained. The correct balance of

the small GTPases (RhoA, Rac1) is important for junctions to be maintained rather than destabilised. Cadherins can direct cell–cell contact in three ways: 1) by reducing the local interfacial tension directly through adhesion tension and 2) indirectly through signalling to the actomyosin cytoskeleton, and 3) by establishing the mechanical coupling of contacting cells. It has been shown (in astrocytes, fibroblasts and endothelial cells) that AJs which form lateral contacts between adjacent leading cells are not fixed, but rather continually undergo retrograde movement from the front towards the cell rear. The speed of the retrograde movement of AJs is independent of the speed of migration and also occurs in non-migrating cells which have a symmetric distribution of AJs. In follower cells, which have no contact-free edges, AJs remain essentially stationary, and an asymmetric distribution of AJs (which results in subsequent cell polarisation) is sufficient to promote a polarised dynamics of AJs, suggesting that during collective migration, the anisotropy of cell–cell contacts in leader cells triggers a highly dynamic retrograde flow of AJs. In the same system, it has been shown that N-cadherin which form AJs also undergo a continuous tread-milling along the lateral sides of adjacent leading cells. This highly dynamic process is controlled by the polarised regulation of p120-ctn and allows for stabilisation and rapid changes in cellular interactions during collective migration (Peglion, Llense, and Etienne-Manneville 2014). The strength and stability of cell–cell contacts is modulated by the cytoplasmic components of the AJ which regulate the adhesive action of the extracellular domain of cadherins in various ways.

Cadherins in CCM

Although the importance of cadherins in adhesion is clear, their role in collective cell migration is not always so straightforward. In epithelial tissue, cadherins (E-cadherin and VE-cadherin), form stable contacts between cells. The stable contacts facilitate collective migration as sheets, strands or isolated groups (Li et al. 2013), e.g. blood

vessel sprouting relies on VE-Cadherin, posterior Lateral Line Primordium of the Zebrafish express several Cadherins and directional migration of expanding cell sheets in 2D-cultures (Theveneau and Mayor 2012). Loss of function experiments (inhibition of cadherin) targeting these molecules impair migration and increases migration randomness. However, even within the epithelial cell population, the role of Cadherin-based junctions during collective cell migration is extremely diverse. Cadherins can be used to; transmit signals via local stress and tension, polarise cells by restricting the formation of protrusions away from the contact, and promote interaction with surrounding tissues if needed (Halbleib and Nelson 2006; Maître and Heisenberg 2013)

In mesenchymal cells, which arise from epithelial–mesenchymal transition (EMT, cells typically lose stable cell-cell junctions before migration (Kalluri and Weinberg 2009). N-cadherin is the most common cadherin found in mesenchymal cells and forms transient adhesion junctions which are important in cell migration, development, cancer metastasis and cell differentiation (Lamouille, Xu, and Derynck 2014). Pre-migratory neural crest cells undergo EMT to become migratory neural crest (NC) cells (Scarpa et al. 2015). This transformation is accompanied by a switch of E to N-cadherin and these mesenchymal NCs exhibit localised N-cadherin at contacts, which are important in the transient cell-cell adhesion process of contact inhibition of locomotion (see next section). These cells undergo collective cell migration using CIL, where transient cell-cell adhesion are overcome and disassembles by the polarised forces, although it is not fully understood how adhesion junctions disassemble during CIL. When cells come into contact, they become transiently coupled via the adhesion junction, resulting in retrograde actin flow decreasing in the region behind the adhesion, which in turn leads to actin stress fibres and microtubule formation which act to further couple the cells. Thus in these cells, N-cadherin acts to increase cell migration, albeit via CIL, whereas E-cadherin suppresses migration by suppressing CIL. When two neural crest cells col-

lide, RhoA activity increases and Rac1 signalling decreases mediated by N-cadherin and Wnt/PCP signalling. This results in lamellipodium collapse. However, unlike in epithelial cells, the protrusions do not spread laterally but instead reform on the opposite side of the cell. Furthermore, the increase in RhoA activity does not lead to the formation of actin bundles parallel to the cell cortex as in epithelial cells. Instead of the cell junction stabilising, the cells undergo contraction via RhoA/Rock signalling to move away from each other.

Ephrins and Contact Inhibition of Locomotion

Contact inhibition of locomotion

Contact inhibition of locomotion (CIL) is the term given to the process where cells change their direction of motion upon coming into contact with another cell (Abercrombie and Heaysman 1953; Abercrombie and Heaysman 1954). Quantitative analysis of CIL is challenging because head-on cell-to-cell collisions are infrequent in traditional cell culture and difficult to isolate in collective migration experiments. The role of CIL during collective cell migration remains unclear, as in some system (epithelial motion) it has never been observed while in others (neural crest) it plays an essential role. It has been shown that in some cases, during collective cell migration, CIL ensures the absence of protrusions at the cell–cell contacts between leading cells and followers, while simultaneously promoting the formation and maintenance of protrusions in the leader cells in a direction away from their contacts with follower cells. CIL has been observed experimentally both *in vitro* (Mayor and Carmona-Fontaine 2010), and recently *in vivo* (Carmona-Fontaine et al. 2008). Furthermore, in many *in vivo* examples of CCM, large protrusions are observed at the leading edge, pointing away from the contact with follower cells. This feature is the hallmark of cell polarisation induced by CIL. Therefore, CIL between leader and follower cells seems to be a fundamental aspect of collective cell

migration. Although CIL is associated with the concept of cell repulsion, it can also be considered a spatial guidance cue, as it results in repolarization and redirection of migration. During CIL, cells first detect other cells, information that the cell has come into contact with another cell is then relayed inside the cell, and finally, intracellular signals drive protrusion collapse at the cell contact (and inhibition of new protrusion formation there) and repolarization. During repolarization, new protrusions are formed away from the point of cell-cell contact. There are many cell surface molecules which are known to be involved in CIL (Mayor and Carmona-Fontaine 2010); these include cadherins (N-cadherin and cadherin 11), ephrins and EPH receptors, members of the planar cell polarity (PCP) pathway, and syndecan 4. Cadherins mediate homophilic interactions and ephrins mediate the heterophilic interaction between colliding cells, via their extracellular domains. Cells can exhibit both homotypic CIL and heterotypic CIL. It is likely that the diversity of surface molecules involved in CIL means cells can use various molecules to interact with their neighbours, giving a greater degree of versatility to CIL.

Ephrin Functions

Signalling between Eph receptors and their ligands, known as ephrins, has emerged as a principal mode of contact-dependent communication between cells and has been found to be important in the regulation of many processes during both embryonic development and adulthood. These include neural development, angiogenesis, border formation and tissue patterning, cell migration, cell morphogenesis and axon guidance (Kullander and Klein 2002). Both ephrins and their Eph receptors can transduce signals (bi-directional signalling), and they are important regulators of cell–cell communication in processes such as cell adhesion and repulsion (Villar-Cerviño et al. 2013; Arvanitis et al. 2013). The repulsive behaviour of Eph/ephrin signals has been well characterised in axonal guidance mechanisms, and it is possible that the repulsive

mechanisms in axon guidance are similar at the molecular level to those controlling protrusion collapse in CIL. At the molecular level, many of the signalling pathways downstream of Eph/ephrin signalling are involved in regulation of the cellular cytoskeleton. In addition to regulating the activity of cytoplasmic modulators of the cytoskeleton and adhesion (Wilkinson 2003; Klein 2012), there is evidence that Eph-ephrin activation can regulate gene expression (Takasu et al. 2002; Lai et al. 2004; Parrinello 2010).

Ephrin Structure

Ephrins are a family of transmembrane proteins which bind to their partner receptor tyrosine kinase Eph. The extracellular domain of Eph receptors is made of a highly conserved globular ephrin ligand binding domain, a cysteine-rich region and two fibronectin type III domains (“Crystal structure of an Eph receptor-ephrin complex.”). The cytoplasmic domain of Eph receptors contains a juxtamembrane region with two conserved tyrosine residues, a tyrosine kinase domain, a sterile alpha motif, and a PDZ-binding motif. In the absence of ligand binding, the unphosphorylated tyrosines near the cell membrane of the Eph receptor bind tightly to the kinase domain, distorting it and preventing tyrosine kinase activity. Upon interaction, there is high-affinity binding between Eph receptors and ephrin ligands, leading to activation of the Eph receptor and signalling on the cytoplasmic side. Following ligand interaction, Eph receptors cluster together and become phosphorylated on the tyrosine residues in the juxtamembrane region of the Eph allowing the intracellular tyrosine kinase to convert into its active form and subsequently activate or repress a variety of downstream cascades in a cell and context-dependent manner. There are two classes of Ephrins (termed A and B) (Lisabeth, Falivelli, and Pasquale 2013), which usually interact with EphA and EphB receptors, respectively, although cross signalling can occur. Ephrin/Eph signalling can activate Rho GTPases, such as RhoA (usually via ephrinA/EphA binding) and Rac1

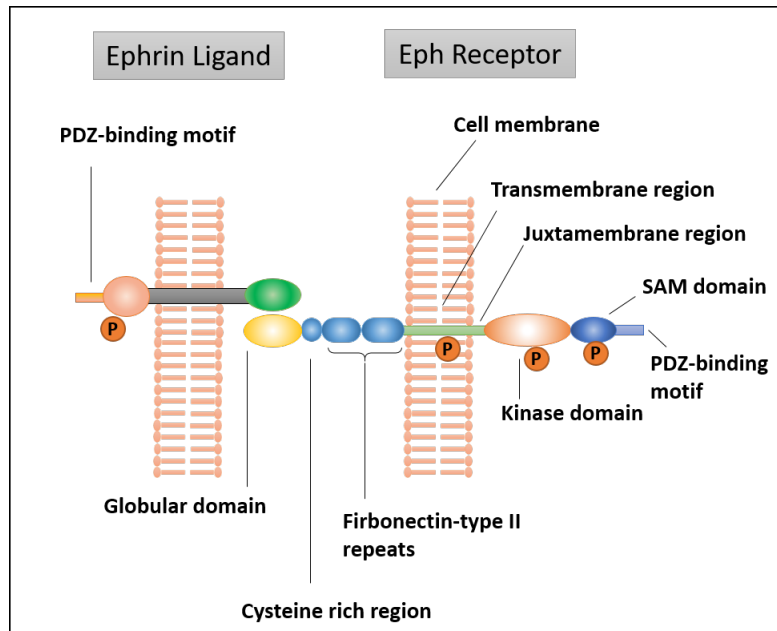


Figure 1.4: Ephrin schematic

and Cdc42 (usually via ephrinB/EphB binding), and EphrinB/EphB can also activate RhoA via Dsh/PCP signalling. Molecules involved in the initial cell–cell contacts are also typically involved in the regulation of small GTPases (RHOA, RAC1 and CDC42), via the activity of the exchange factors TRIO and VAV2, which in turn direct cytoskeletal and actin dynamics.

CIL and Adhesion

Although it may appear contradictory, there is, in fact, an established link between CIL and adhesion (Abercrombie and Ambrose 1962). There is evidence which suggests that molecules which are usually linked to cell–cell adhesion, are likely to mediate CIL. Although CIL usually results in cell repulsion and dispersion, cell–cell adhesion is a necessary step in this process. It allows for strengthening of the contacts between cells, which in turn allows cell–cell signalling to occur. Establishment of transient adhesion points between colliding cells has been observed *in vitro* preceding lamellipodia

retraction and repolarization via CIL. Furthermore, adhesion molecules do not only support mechanical adhesion, but they also can play a significant role in cell signalling. For instance, N-cadherin is specifically required for mediating contact inhibition of locomotion in neural crest cells (Mayor and Carmona-Fontaine 2010; Becker, Mayor, and Kashef 2013). N-cadherin at cell contacts inhibits protrusion and downregulates Rac1 activity and in turn, promotes protrusions and activation of Rac1 at the free edge. N-cadherin thus stabilises contact-dependent cell polarity, resulting in directional collective migration. (Theveneau et al. 2010). In neural crest cells, overexpression of E-cadherin abolished CIL and induced an epithelial pattern of contact regulation, even though N-cadherin is present.

1.5 Peripheral Nerve Repair

The peripheral nervous system (PNS) is a network of motor and sensory nerves that connect the brain and spinal cord (the central nervous system) to the entire human body. These nerves transmit electrical impulses from the central nervous system (CNS) to the body, controlling the functions of sensation, movement and motor coordination. Peripheral nerves, in contrast to those in the CNS, have the remarkable ability to regenerate and re-establish function after injury (Nguyen, Sanes, and Lichtman 2002; Navarro 2009; Zochodne 2008).

The nervous system is composed of two broad categories of cells, neurones and glial cells. Neurones are responsible for electrical transmission of impulses and information processing, while the glial cells are the support cells of the system. Nerves are composed of bundles of neurones, and each nerve is made of three layers, an inner layer, the inner endoneurium, which covers the nerve fibres (axons); a middle layer called the perineurium, and an outer layer over the perineurium, called the epineurium. The

major glial cells of the peripheral nervous system are Schwann cells. They encircle the nerve fibres (axons) and can be either myelinating or non-myelinating. Myelinating Schwann cells insulate the axons, enabling the rapid transmission of electrical impulses essential for nervous system function. Non-myelinating Schwann cells usually have metabolic and mechanical support functions, maintaining appropriate concentrations of ions and neurotransmitters in the neuronal environment (Jessen 2004). In addition to these roles, Schwann cells play an important positive role during nerve repair after injury.

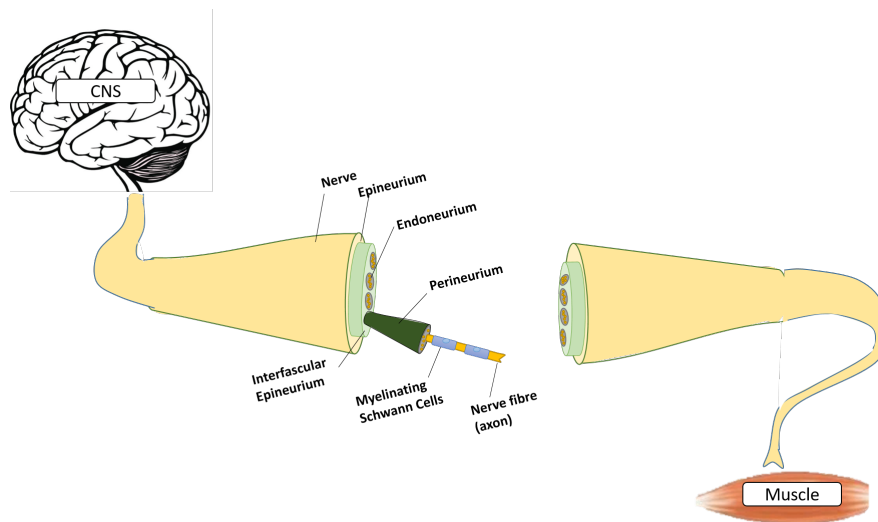


Figure 1.5: There are three main types of nerves: Afferent nerves, efferent nerves and mixed nerves. Afferent nerves transmit signals from sensory neurones to the central nervous system; efferent nerves send signals from the central nervous system to the muscles and glands, and mixed nerves are responsible for receiving sensory information, and for transmitting information to the muscles.

Regeneration in the PNS following crush injuries is usually very successful since the basal lamina which surrounds the axon is preserved, conserving the integrity of the original axon process paths and thus enabling highly efficient and successful target re-innervation (Nguyen, Sanes, and Lichtman 2002). However, regeneration can also occur after full nerve transection (i.e. after more severe injuries). In this case, the

repair process is less efficient as the transection means there are many more challenges which must be overcome for successful repair to occur (Nguyen, Sanes, and Lichtman 2002). Upon being cut, both nerve stumps retract to form a gap (the wound gap), which must be bridged by new tissue. Regrowing axons from the proximal stump need to travel through this tissue (the nerve bridge) in order to reach the distal stump and to reinnervate their target organs (Zochodne 2008). A number of complex processes occur to facilitate this, and Schwann cells play an important role. Nerve repair can occur successfully if the cell body of the neurones is undamaged and remains intact, if scar tissue does not form too quickly and if the Schwann cells act in their reparative capacity.

After transection nerve injury, axons distal to the injury site degenerate. During this process, Schwann cells undergo a remarkable transformation to stem-cell like cells. In the proximal nerve stump, Schwann cells de-differentiate, temporarily losing their specialised myelinating function and adopt the phenotype similar to that immature become Schwann cells, with some additional features (Jessen, Mirsky, and Lloyd 2015). Myelin is downregulated, while proliferation promoting genes, and those encoding cell adhesion molecules, growth factors and their receptors, are up-regulated. Schwann cells then proliferate and migrate out of the proximal stump into the wound gap where together with macrophages they remove inhibitory debris from the wound site thus creating a favourable environment for repair (Toy and Namgung 2013). Schwann cells which migrate into the wound site express EphB2 which interacts with ephrinB2-expressing fibroblasts, activating a signalling cascade in Schwann cell leading to the transcription factor Sox2 accumulating in the nucleus. Sox2-mediated transcription results in re-localisation of N-cadherin to Schwann cell-cell contacts which lead to the formation of Schwann cell cords, which serve as a substrate to support the regrowth of axons (Parrinello 2010). These cords serve as guidance conduits, known as bands

of Büngner, which aid repair and regeneration by providing a path and scaffolding for regrowing axons (Scheib and Höke 2013). These scaffolds/guidance conduits are essential for successful repair in transection injury since, unlike in crush injury, the majority of the structure of the nerve and the Schwann cell basal lamina are lost.

Thus, after injury in the PNS, although axons may degenerate, in time, new projections start to regrow back from the proximal to the distal stump and eventually reconnect to their target organs to re-establish nerve function. Once the neurones have reached their targets, the Schwann cells then stop dividing and re-specialise to insulate the newly reformed nerve. Significant work has been done into injury-induced Schwann cell reprogramming (Jessen and Mirsky 2016). However, less is known about the way in which Schwann cells migrate and form the cords which guide the axons across the wound site (Parrinello 2010). Previously work in the lab has shown that cord formation depends on the relocation of N-cadherin to cell-cell contacts downstream of Eph signalling (Parrinello 2010). However, the exact mechanisms by which this occurs and the precise effect it has on collective migration remains elusive. Furthermore, it remains unclear how these changes result in directional collective migration.

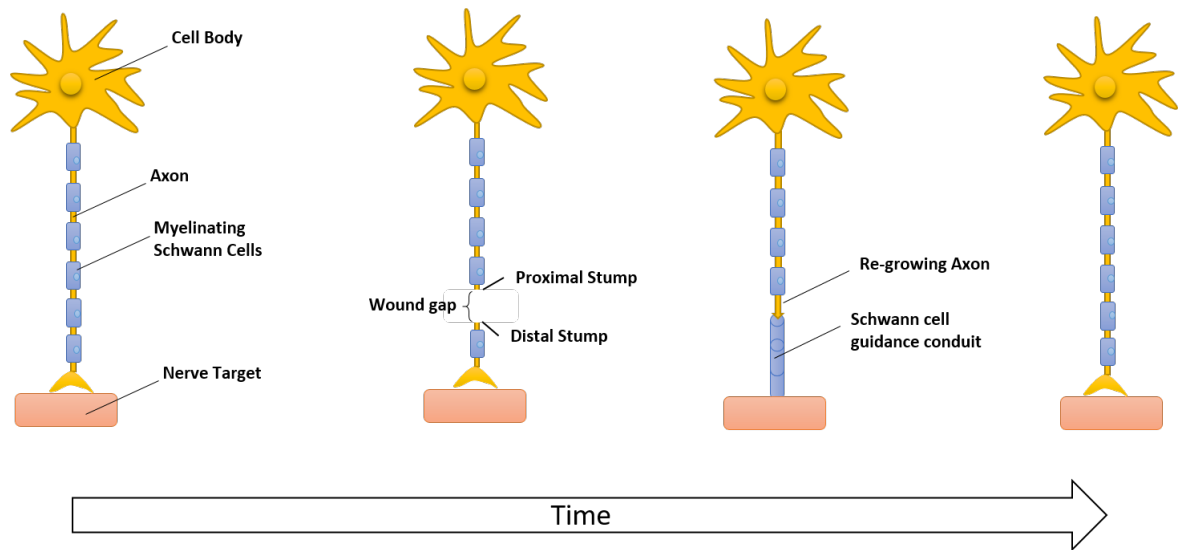


Figure 1.6: Schematic of the axon repair process

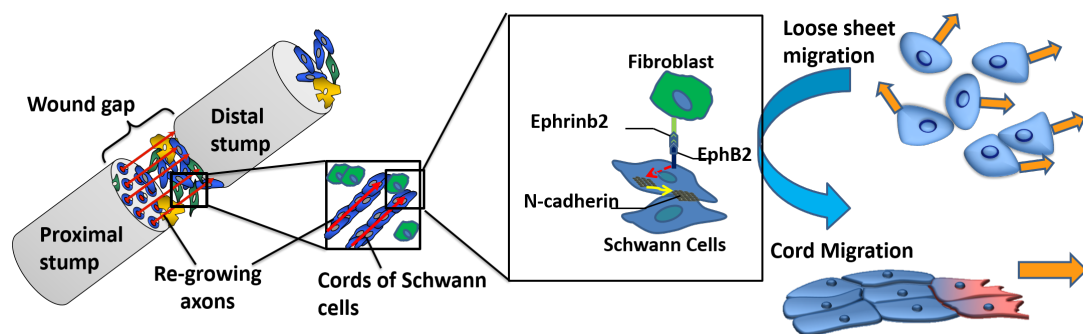


Figure 1.7: Graphical summary of what is known about the role of Schwann cells and fibroblasts in peripheral nerve repair.

1.6 Modelling Collective Migration

Computational models are excellent tools with which to study complex biological systems. In combination with laboratory experiments, computational models allow for hypotheses testing for experiments which would be too time-consuming, costly or impossible to carry out. They allow exploration of system behaviour and exploration of experimental design space which can aid understanding of non-trivial systems. Theoretical modelling approaches have already provided valuable insights into collective cell migration, although the majority of models have focussed on single cell migration modes. For example, models can describe intracellular actin remodelling dynamics and single-cell mechanics during migration (Woods et al. 2014). Models cannot replace experiments, nor are they sufficient alone to prove that particular mechanisms are at work in a given situation. Nevertheless, they can show whether or not a proposed mechanism is enough to reproduce a phenomenon we observe experimentally (Brodland 2015).

Previously, collective cell migration was mainly investigated by experimentalists and the results and analysis focussed on the phenomenological aspects. However, more recently, the use of quantitative methods has become more mainstream. This approach is useful as it allows for numerical comparison between different systems, between perturbations to a system and for the accurate characterization of features in different systems. It is, therefore particularly suitable for the elucidation of the roles of different and often antagonistic behaviours which are present in collective cell migration. A quantitative approach is also essential for the comparison of data from *in vitro* and *in silico* experiments. Without a readout of the accuracy of the results of simulation or equation, concerning the real life data that is being modelled, these approaches have little or no value. It is important also to use measurements which are relevant and

meaningful to the system in question. Models are typically general or specific to the system. The more general models are based on simple rules required for the emergence of collective motion while more specialised models include other interactions unique to the particular experimental situation. The models can replicate the collective motion patterns similar to many of those observed in experiments. Using quantitative measures, objective comparisons between experimental and computation data can be made. These models can be used to interpret phenomena like cell sorting and collective motion. Since these models are based on only a small number of terms, they cannot account for the numerous potential factors that may influence the precise cell motion. However, this approach can be used because the details are averaged out when the behaviour of the whole is considered.

There are three main approaches for *in silico* modelling of collective cell migration. These are; continuum models, discrete models and models which combine elements from both discrete and continuum models. Continuum models use a macroscopic approach and describe the average behaviour of cell populations using continuum variables. In continuum models, cells are represented as populations which have associated properties which are defined as averages for the whole cell population. This type of modelling is useful population scales where modelling each cell as a single entity can be computationally prohibitive. Continuous models are deterministic and are described using differential equations. The main advantages of using this type of model are; (1) it is well-established and thus well understood, (2) the analysis of these systems is relatively simple compared to other types of models, and their solutions can be computationally inexpensive to calculate. However, the drawback of continuum models is that they do not capture spatial dynamics or stochastic effects and they require much more assumptions than discrete models and are very theoretical. Continuum models have been used in modelling cell colony expansion in wound healing and cell

adhesion in cellular invasion processes in both cancer and development (Gerisch and Chaplain 2008; Painter, Armstrong, and Sherratt 2010; Arciero et al. 2011).

Discrete models where interaction rules are described at the cellular scale. In these models, individual agents interact with the environment and other agents according to a set of rules and make decisions that lead to a particular behaviour. Discrete models can incorporate stochasticity; for example, an agent can update its location at a given probability instead of following a deterministic process. This is particularly useful since stochasticity is known to play a major role in biological systems. These models are useful as they can deal with multiple scales at the same time, enabling analysis of emergent behaviour at the population from local interaction individual cells at lower scales. The downside of these models is there is a high computational cost, and models require hours or even days to run a single simulation. Moreover, stochastic models need to be simulated numerous times to obtain the overall average behaviour of the system. Therefore, these models are often only used when the number of individual agents is limited to a small number. These models can produce high-level, emergent phenomena. Furthermore, these models can be increased or decreased in complexity easily, and multiple types of agents can be included to allows exploration of complex behaviours.

Cellular automata (CA) are one of the most commonly used models. They are based on a lattice of points (which represent cells), each of which can be in a finite number of states, represented by one or more discrete or continuous variables. Each cell is connected to a finite number of neighbour cells, whose states at time t_n result in a new state at time t_{n+1} . The evolution of the agent's state and their position is controlled by a set of rules, and both the current state of the agent and the states of the surrounding agents are used in these rules. Alternatively, off-grid models can be utilised, where cells are described as state vectors or position, velocity and state. Depending on the

level of complexity or scale of the model, discrete agent models can be nuclei-centred models or deformable cell models. In the nuclei-centred models, cells are represented as locations in space, with a fixed area of occupation, whereas the deformable cell models represent cells as a deformable shape and area (or volume) which can change in time depending on the cell state as well as on the interaction with surrounding cells. In these models, cells interact with neighbouring cells within a given interaction radius.

Using models like these, fundamental characteristic patterns of motion in collective cell migration can be replicated and identified. These include: 1) disordered motion (direction of movement is not correlated), 2) ordered motion (even distant cells move in a similar direction), 3) turbulent motion (local order which is lost on a scale larger than the size of the units), 4) “streams” of units flowing opposite to each other and finally, 5) “jamming” when the restricted volume and mutual pushing of cells result in a strained, locally fluctuating, globally stationary groups of cells. The extent of collective motion of a population can be measured by choosing some suitable order parameter. Thus, these behaviours can be evaluated using this order parameter and studied how they arise in conjunction with the parameters of the system, for example, cell adhesion, polarisation, or density.

2

Methods

2.1 Introduction

To study Schwann cell-fibroblast interactions and Schwann cell collective migration at a cellular level we use an assembly of cell and molecular biology techniques. In this chapter, I describe the protocols I use to extract Schwann cell features and characteristics, collective migration assay, data extraction methods, and some of the quantitative methods I use in my analysis. Methods for the quantification of the effect of fibroblasts on Schwann cells is described in Chapter 3. Methods for the quantification of collective cell migration is described in Chapter 4. Computational modelling methods and

parameter fitting methods will be described in Chapter 5.

2.2 Cell Culture

Primary rat Schwann cells and fibroblasts were cultured from P7 animals as previously reported (Mathon et al., 2001). Cells were used up to passage P20.

2.3 Assays

Cell Sorting

Schwann cells were seeded at $7.5 \times 10^4/\text{cm}^2$ on recombinant ephrinB2 ($8\mu\text{g}/\text{ml}$) and PLL-laminin coated cover slips in Schwann cell medium. Ephrin coating is made by covering PLL coated glass coverslips in a solution of PBS, ephrinB2 and clustering anti-Fc antibody. Control coating contained no ephrinB2. Cultures were analysed 24 hr later unless otherwise specified.

Cell Proliferation Assay

Schwann cells were seeded at $7.5 \times 10^4/\text{cm}^2$ on PLL-laminin coated cover slips in Schwann cell medium. Once attached, cells were incubated for 6hrs ($2\mu\text{M}$) with Edu-Click. Cells were fixed using 4% PFA.PBS (15 minutes, room temperature), washed 3 times in PBS and incubated with Click-iT Plus for 30 mins.

Cell Motility Assay: Boyden Chamber

Schwann cells were seeded into the upper chamber in serum-free media (1.5 ml), and conditioned media from fibroblast was placed into the lower one (1ml) 3.21A. Cells were seeded at a concentration of $40 \times 10^3/\text{ml}$. The pore size of the filter is large

enough to allow for chemotaxis of the Schwann cells (millipore $8\mu\text{m}$). The filter was coated with laminin ($10\mu\text{g}/\text{ml}$). The Schwann cells were incubated overnight at 37°C , 5%, CO_2 , 95%. The following day, Schwann cells were fixed (0.5% PFA solution) and migration of Schwann cells through the membrane was quantified by staining Schwann cells with crystal violet (0.05% solution) and manually wiping away cells on the upper side of the membrane (which was first observed to check equal numbers of cells were able to attach to the membrane) 3.21B. Images were taken of the stained membrane, and migration was quantified as a percentage occupied by the total membrane area. This is an accurate enough method for when large changes are observed (as is the case here). More sensitive methods of migration can be used (for example a plate reader). However, it was not necessary for this situation (Hong-Chen 2004). Experiments were done a minimum of three separate times, with triplicate biological repeats on each occasion.

To make fibroblast or Schwann cell conditioned media cells were seeded in 10cm tissue culture plates at 5×10^5 and allowed to grow until confluent. Once confluent, cells were washed three times with PBS and incubated in 10ml of conditioning media (low of 0.2% serum) for 24hrs. To make conditioning media $10\mu\text{l}$ / glutamate, 1.2ml Kan/Gen $50\mu\text{l}$ transferin, $100\mu\text{l}$ insulin and 50ml SATO were added to 500ml of DMEM.

Collective Migration Assay

Schwann cells were placed in a hanging drop culture and incubated under physiological conditions until they aggregated under gravity and formed tight adhesions. Schwann cells are spun down (2500 rpm, 3 minutes), the supernatant is removed, and the pellet is re-suspended in a small volume of Schwann cell media (1×10^6 cells/ $30\mu\text{l}$ drop). A drop of $30\mu\text{l}$ is pipetted onto the underside of a small tissue culture lid. A small amount of media or PBS is placed in the bottom of the dish, to act as a hydration chamber. The lid is placed onto the PBS-filled bottom chamber and incubate at 37°C , 5%, CO_2 , 95%

humidity, until the cells aggregate (usually 5-6hrs).

On laminin and PLL coated coverslips, a layer of 0.5% matrigel is added by using 180 μ l per coverslip. Small clumps of Schwann cells were plated by using a 10 μ l pipette to detach and pick up a small section of the aggregate (roughly 2 μ l). The small clump is placed in centre of the cover-slip/plate, and a small amount of SC media is added so the cells are fully covered. The cells are incubated for half an hour, and then more media is added (0.5 ml). Schwann cells took about 3hrs to fully adhere from this point. Once Schwann cells were attached, fibroblasts can be added, the number of fibroblasts used is 35×10^3 /well, unless stated otherwise.

Live Cell Imaging The image sequences were obtained using an imaging workstation, composed of an inverted microscope equipped with a temperature, humidity and CO₂ controlled chamber and camera connected to a computer and used with Velocity Software. Images were taken at intervals of 10 minutes (unless stated otherwise) for several days. Primarily phase contrast images were recorded, however on occasion Schwann cells expressing GFP or labelled with a fluorescent marker (eg CellTracker) would be used and fluorescent images were also recorded. Images were recorded using a x10 lens, and since the region of interest recorded from a single frame was too small for our purpose, multiple images around a region of interest (ROI) were recorded and stitched together to form a larger composite image. The data recorded from these experiments were then exported as stacks of images, for analysis in MATLAB.

Cadherin Quantification Method

To quantify the re-localisation of N-cadherin, Schwann cell were stained for antibodies against N-cadherin. All cells were also stained with Dapi to facilitate calculating cell density within the region of interest and Schwann cells were labelled with cell-mask so that Schwann cells could be identified. The amount of N-cadherin localised at the cell-

cell boundaries was calculated using a custom thresholding method in MATLAB. The total amount of N-cadherin was then calculated by summing the pixels and normalising by the number of cells.

Quantification of Contact Inhibition of Locomotion

Quantification of CIL was carried out by measuring the displacement of a migrating cell for 30 min before collision (vector A) and for 30 min following collision (vector B) was measured. The component Cx of vector B–A represents the difference between how far the cell has progressed in the direction of A' the distance it would have moved had there been no collision. Cx values were also calculated for the same population of cells which were able to move freely without collisions over the same time periods. CIL was considered to have occurred when the mean Cx value of free-moving cells was significantly different to that of colliding cells. The centre of the cell was used as a marker to track vectors A and B. Only collisions of leading edge lamellae were analysed and collisions, where more than two cells were involved, were excluded. Cx measurements were scaled to normalize for differences in speed between cell populations (Batson et al. 2014).

Nuclei Detection Nuclei in fixed cell cultures were detected using the Dapi fluorescent signals. In some cases when cells were highly clustered

LifeAct Cells were seeded at 150×10^3 cell/well (6 well plate) in Schwann cell media and incubated overnight (37 °C). The following day cells were infected with LifeAct virus (1:30) and incubated overnight.

Antibodies Primary antibodies for Western blotting and immunofluorescence analysis were: anti-RT97 (kind gift of J. Woods), anti-neurofilament(Abcam), anti-S100b (DAKO), anti-p75NGFR (Chemicon), anti-fibronectin (Sigma), anti-prolyl-4-hydroxylase b (Acris), N-cadherin (BD transduction labs), anti-EphB2 (R&D systems),

anti-Sox2 (kind gift of M. Wegner).

3

Characterising Schwann Cell and Fibroblast Interactions

3.1 Introduction

Characterising how Schwann cells change in response to interaction with fibroblasts allowed me to understand better how fibroblasts can impact Schwann cell migration. I first studied sparsely plated Schwann cells (low density), to elucidate the effects of fibroblasts on individual Schwann cells. Using different cell culture systems in conjunction with time-lapse video recording, metabolic labelling and RNAi knockdown,

I found nerve fibroblasts differentially modify the various responses of Schwann cells. Since I was interested in the role of ephrinB2, where possible experiments were performed using fibroblasts or using ephrinB2 coating in place of fibroblasts, to investigate whether ephrinB2 could replicate the effects of fibroblasts.

Although collective migration is more complex than just many individual cells moving in the same direction, having a clear understanding of the phenotypes and behaviour of individual cells is very useful in a) interpreting results at the population level and b) building a computational cell-scale model based on phenotypes. Moreover, using quantitative metrics, rather than qualitative observations, helped to increase the understanding of the system, but it is also essential for determining which features should be included in the *in silico* model. In this chapter, the results of the effect of fibroblasts on Schwann cell morphology, proliferation, motility, clustering and alignment are reported.

3.2 Schwann cell migration in sparse setting

To examine whether fibroblasts influenced the migration speed or mode of Schwann cells, the migration of individual Schwann cells was analysed by time-lapse video recording over a 48-h period (see Fig. 3.1 for representative snapshot). Schwann cells were labelled with cell-mask and were plated sparsely (to minimise cell-cell interaction and allow the space for migration) alone or in a co-culture with fibroblasts (1:1 ratio). Phase contrast and fluorescent images were recorded at 10-minute intervals at x10 magnification. A 10-minute interval was chosen to avoid the measurement error of short time scale analysis - briefly, this kind of error can arise when the human error in selecting the cell location can be larger the actual movement. The analysis was carried out in the period between cells attaching properly and before the cell density

increased (due to proliferation) to minimise the effect of lack of space. Consecutive frames were loaded into MATLAB, and cells were tracked by manually clicking to label the centre of mass of the cell at each time point. Their migration trajectories, speed cell morphologies were analysed in MATLAB.

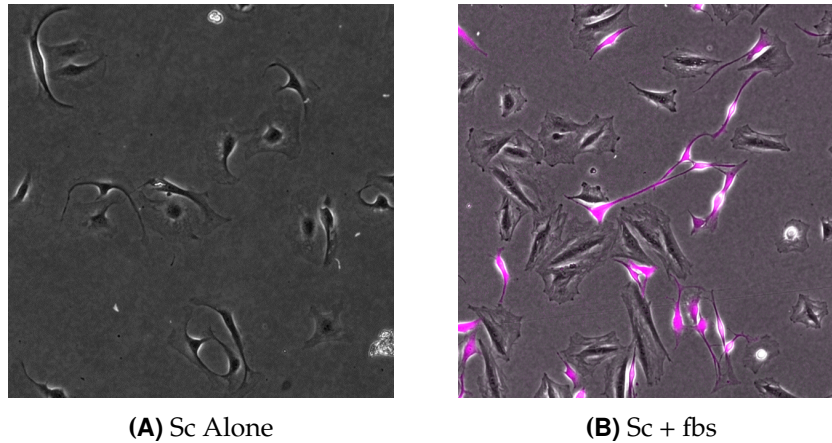


Figure 3.1: Zoomed in snap shots of (A) Schwann cells alone cultured alone and (B) Schwann cells in co-culture with fibroblasts - in the co-culture, Schwann cells are labelled with CellTracker (here false coloured in pink) to differentiate between Schwann cell and fibroblasts.

Schwann cell morphology and motility changes when co-cultured with fibroblasts

Phase contrast images (as shown in Fig. 3.1), showed that Schwann cell modes of motility were complex and an individual Schwann cell could exhibit multiple distinct motile phenotypes. 100 cells were studied from 3 separate experiments. Broadly; when Schwann cells were cultured alone, their morphology was typically either (1) rounded (non motile) Fig. 3.2A, (2) flat and asymmetric (motile) with one relatively flat and wide extension (leading edge) Fig. 3.2B, or (3) a flat with second thin extension (trailing end) Fig. 3.2C. Schwann cells with a motile morphology moved in the direction of the leading edge and their motion can be described as a random exploration (random walk, no directed motion) Fig. 3.8C, Fig. 3.8E. When Schwann cells were co-cultured

with fibroblasts, they typically exhibited (1) symmetric bipolar Fig. 3.2D, (2) multi-polar phenotypes Fig. 3.2E, or (3) antisymmetric bipolar Fig. 3.2F and their motion was a constrained exploration Fig. 3.8D, 3.8F.

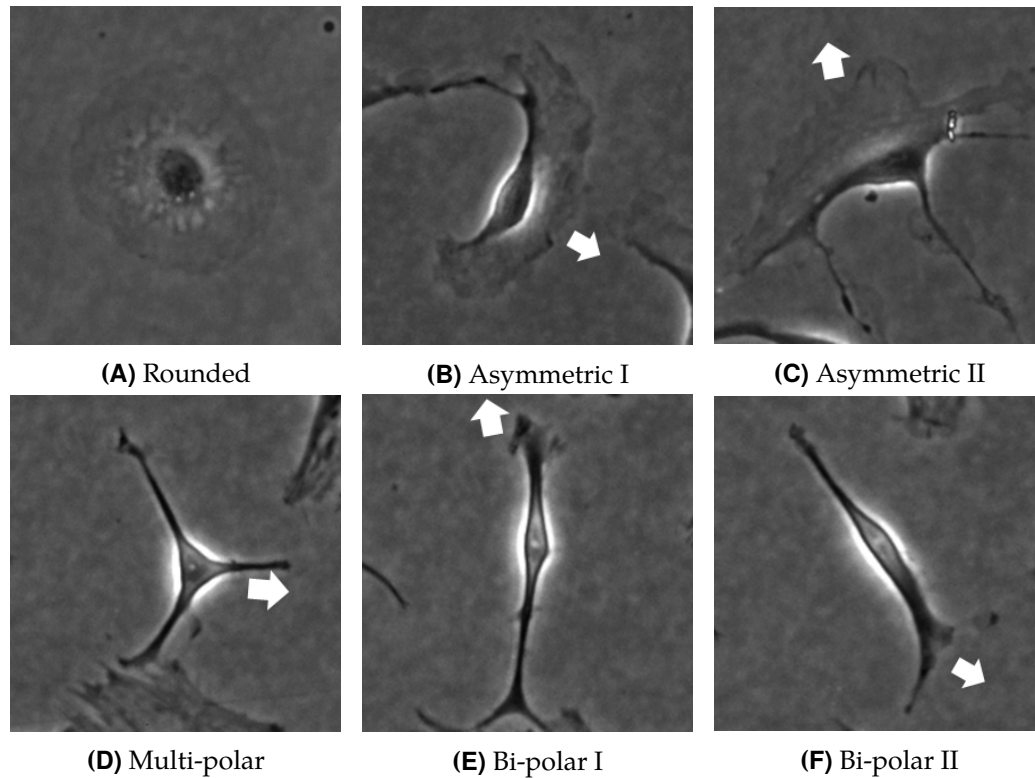


Figure 3.2: Examples of Schwann Cell Morphology: Schwann cells cultured alone (A - C) and co-cultured with fibroblasts (D-F). Schwann cells cultured alone typically exhibit a more rounded or flat morphology, whereas when in co-culture with fibroblasts Schwann cells tend to adopt an elongated, bipolar or multi-polar morphology. White arrows show direction of cell locomotion.

Live cell imaging (LCI) and morphology analysis showed that these morphologies were not fixed. In some cases, an elongated bipolar morphology could be maintained for some time. However, a cell with an elongated morphology would not permanently adopt this shape and would often transition back to a fan shaped/multipolar morphology before becoming elongated again, for example, see Fig.3.3. Since the elongated morphology was not observed in experiments where Schwann cells were in a

mono-culture, this suggested that heterotypic interactions between Schwann cells and fibroblasts, either via direct contact or soluble factors had an impact on Schwann cell morphology. Schwann cells which were part of a cluster of Schwann cells typically maintained a non-rounded morphology, however as can be seen in Fig. 3.3, Schwann cells independent of clusters also exhibited an elongated shape. This suggested that stable cell-cell contacts with other Schwann cells were not necessary for the change in morphology from rounded to elongated.

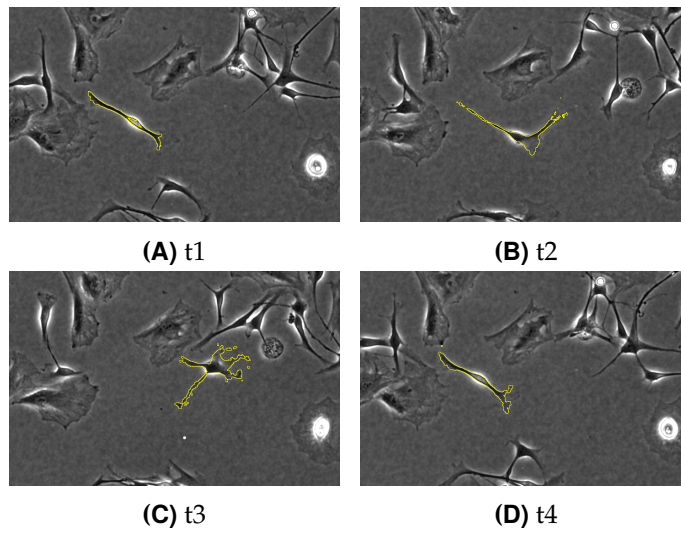


Figure 3.3: Outlines of Schwann cell morphology at successive time points ($dt = 40$ minutes). Schwann cells can be in an elongated morphology while not in contact with other Schwann cells (A, B, D), however they can also lose this morphology and assume a multi-polar morphology (C).

Schwann cell exhibit persistent migration when co-cultured with fibroblasts

When quantifying persistence or randomness in cell migration the time scale is important. At short time-scales, cells appear to move in a persistent direction, while at longer time-scales, the cells (assuming no directional cues) migration can be described as a random walk. For purely random walk, the mean square displacement should increase linearly with time,

$$\langle d^2(t) \rangle = 4\mu t \quad (3.1)$$

where μ is the motility coefficient and $d_i(t) = r_i(t) - r_i(0)$ is the displacement of an individual cell at time t . However, if cells exhibit some persistence (i.e. a persistent random walk), equation 3.1 can be modified to account for persistence in directional migration observed at short times

$$\langle d^2(t) \rangle = 4\mu(t - P + P \exp^{-t/P}) \quad (3.2)$$

where P is the persistence time.

Schwann cells when cultured alone did not exhibit any persistent migration. Their migration mode is a random walk, where the step size is larger for motile cells (fan morphology) and small for non-motile cells (rounded morphology). Examples of cell trajectories are shown in Figs 3.4A and 3.4B. However, when Schwann cells were co-cultured with fibroblasts, their migration pattern exhibited some persistent motion. Cells often adopted an elongated morphology and moved along the direction of the major axis, however when they come into contact with a fibroblast, they undergo contact inhibition of locomotion and retrace their path, moving along a preferential axis (elongated axis) in the opposite direction. Therefore the typical trajectory of Schwann cells cultured with fibroblasts is retraced tracks as shown in Fig. 3.8E. Cells achieve persistent directional migration via the formation and stabilisation of actin-rich protrusions or lamellipodia which maintain the orientation of the leading edge (Andrew and Insall 2007; Arrieumerlou and Meyer 2005). During cell migration, front-to-rear polarisation and cell orientation are thought to be controlled by independent polarity pathways. The small G-proteins (Cdc42, Rac and Rho) are believed to play important roles in the initial signals leading to the polarisation of migrating cells (Charest and Firtel 2007). To quantify persistence in motion, the turning angle at the sampling interval $\Delta t = 10$ minutes was measured (see Fig.3.5).

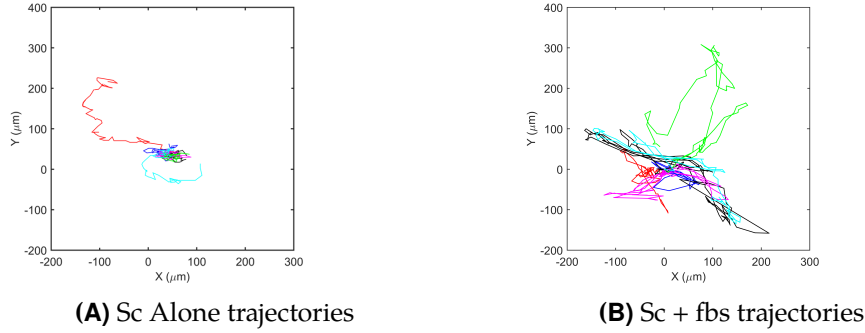


Figure 3.4: Early Time Point Trajectories for (A) Schwann cells plated alone and (B) Schwann cells plated in co-culture with fibroblasts. Cells were tracked early after seeding (once cells has attached) so the low cell density was maintained. Each trajectory has been centred at $x = 0, y = 0$ for comparison.

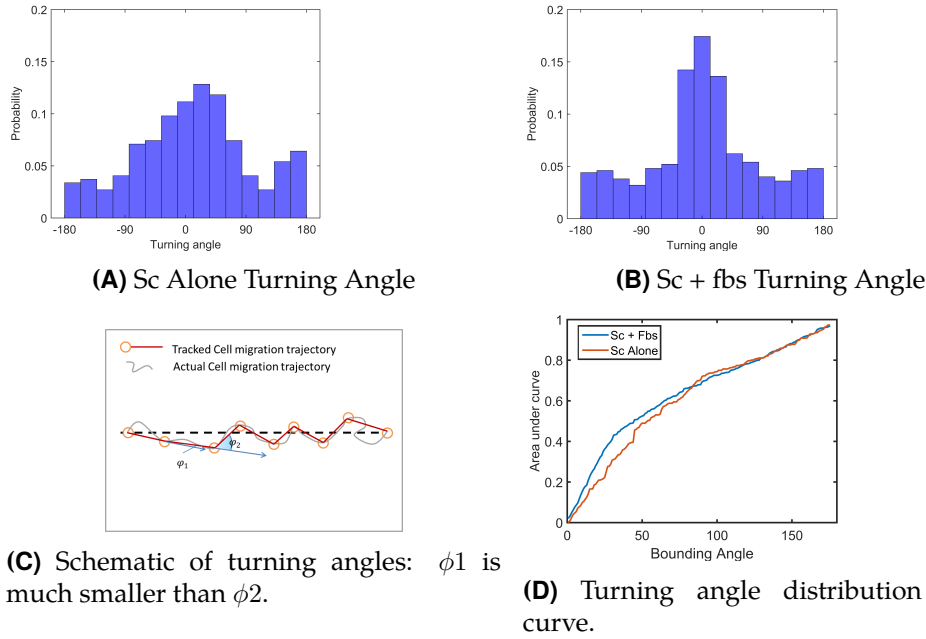
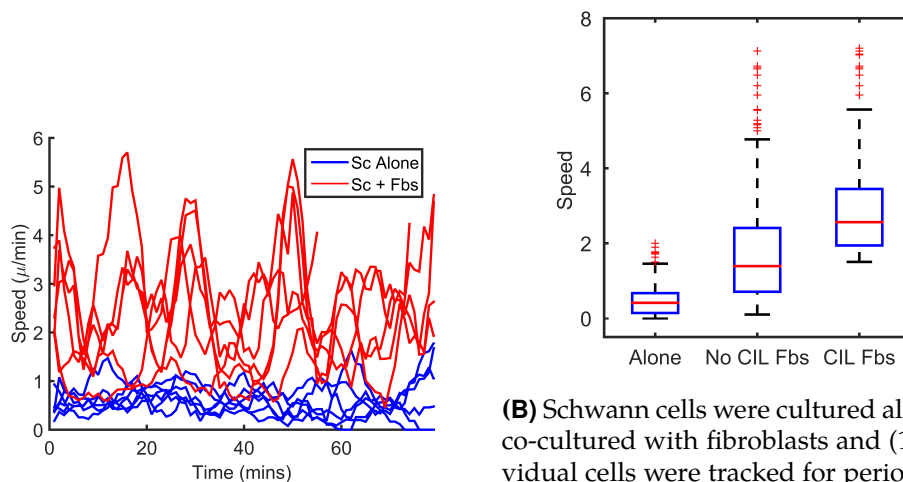


Figure 3.5: Histogram of turning angles for Schwann cells cultured alone (A) and co-cultured with fibroblasts (B). Schwann cells co-cultured with fibroblasts exhibit more persistent motion than Schwann cells cultured alone. (C) Turning angles, ϕ_1, ϕ_2 , are defined as the angle between successive tangent vectors is used as a measure of persistence. (D) The fraction of all turning angles between $\pm$ the bounding angle is shown in the turning angle distribution (TAD) curve.

Fibroblasts increase Schwann cell speed

Schwann cells cultured alone had a relatively constant migration speed, while Schwann cells cultured with fibroblasts exhibited a much more varied migration speed profile (see Fig 3.6A). Schwann cells cultured with fibroblasts had a mean migration speed ($2.33 \pm 1.03 \mu\text{m}/10 \text{ minutes}$) about four times greater than Schwann cells cultured alone ($0.54 \mu\text{m} \pm 0.27 \mu\text{m}/10 \text{ minutes}$). However, the variance in migration speed of Schwann cells in co-culture with fibroblasts was much greater than Schwann cells cultured alone (see Figs. 3.6A and 3.7).



(A) Speed profile of 6 individually tracked migrating Schwann cells when cultured alone (blue), and co-cultured with fibroblasts (red).

(B) Schwann cells were cultured alone and co-cultured with fibroblasts and (15) individual cells were tracked for periods of 45 minutes. The leftmost label (alone) shows the mean speeds of Schwann 15 cells cultured alone. The label: "No CIL, fbs" shows the mean speeds of 15 Schwann cells migrating and no interactions with fibroblasts occur, and the label "CIL, fbs" shows the mean speeds of 15 Schwann cells migrating while interactions with fibroblasts occur.

Figure 3.6: (A) Schwann cells alone have a lower migration speed and smaller variance in migration speed than Schwann cells cultured with fibroblasts. (B) Schwann cells migrate faster when in co-culture (No CIL fbs, CIL fbs) with fibroblasts than when cultured alone (Alone). However, when interacting with fibroblast (CIL fbs), the speed of migration transiently increases.

Schwann cells cultured alone displayed homotypic contact inhibition of locomotion -

upon contact, cells moved away from each other. When co-cultured with fibroblasts, Schwann cells underwent contact inhibition of locomotion with fibroblasts, however, adhered to one another forming clusters. This suggested that the adherens junctions between cells overcame the contact inhibition which was present when they were cultured alone.

Individual Schwann cells were tracked during periods of no interaction (i.e. the Schwann cell had not in the past 30 minutes interacted with either a fibroblast or another Schwann cell). The mean speed of non-interacting Schwann cells in co-culture (i.e. cells tracked during periods of non-interaction with other cells) was significantly higher than when cultured alone (see Fig. 3.6B). Upon interaction with fibroblasts, there was a transient increase in Schwann cell speed. This suggested that the increase in Schwann cell migration speed is due to both soluble factors (i.e. a non-contact dependent mechanism) and a contact dependent mechanism (i.e. contact inhibition of locomotion).

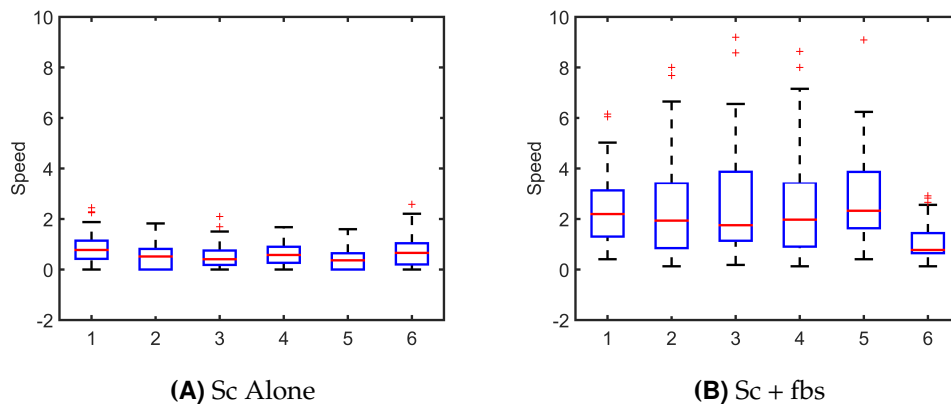


Figure 3.7: Schwann cells were cultured alone (A) or co-cultured with fibroblasts (B). In each case, 30 (5 cells from 6 independent experiments) individual cells were tracked, here box-plots of the cell speed for six representative cells (one from each experiment) are shown. Schwann cells alone (A) have a lower migration speed (across all cells 0.9) and smaller variance in migration speed than Schwann cells cultured with fibroblasts (B).

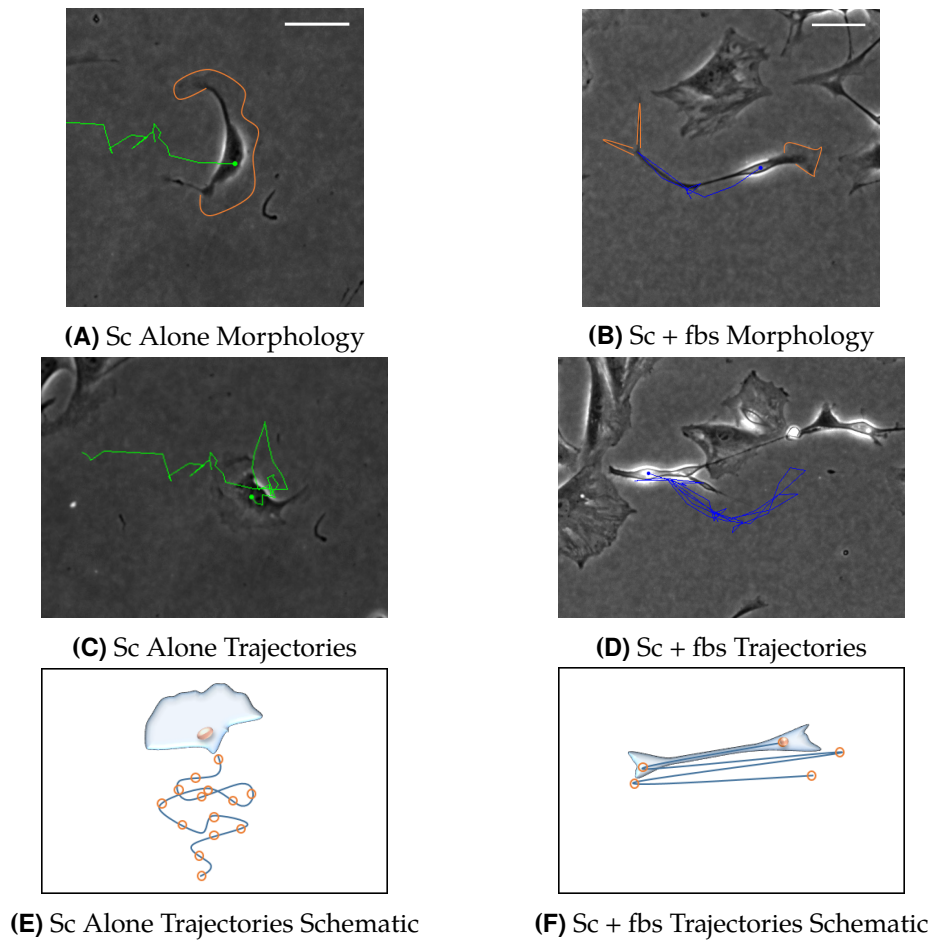


Figure 3.8: Examples of cell trajectories associated with different cell morphologies. When Schwann cells are cultured alone, they primarily adopt a fan shaped morphology, whereas when co-cultured with fibroblasts they adopt an elongated morphology.

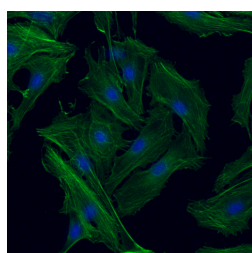
3.3 Impact of Fibroblasts on Schwann Cell Morphology

Impact of Fibroblasts on Schwann Cell Morphology in Co-Culture and on ephrinB2

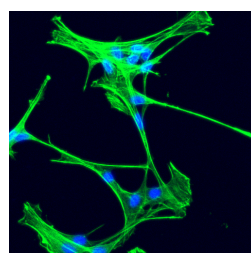
As discussed above, when Schwann cells were cultured alone (on tissue culture plastic) they were primarily rounded, or in a classical fan shaped migrating morphology (see Figs. 3.9A). However, when Schwann cells were co-cultured with fibroblasts or cultured on an ephrinB2 coating, a striking change occurred in their morphology. Schwann

cells became elongated, their morphology became primarily symmetrically bipolar or multipolar and exhibited stress fibres and bundles of cytoskeleton aligned in one direction (see Figs: 3.9B).

As Schwann cells exhibit elongated morphology when co-cultured with both fibroblasts and when cultured on coated ephrinB2, this suggested that ephrinB2/ephB2 signalling played a role in this morphological changes. Cell elongation is typically controlled by internal remodelling of the cytoskeleton and such striking change in morphology suggests ephrinB2 influences the cytoskeleton. It is well known that, via binding to their membrane-tethered ephrin ligands, eph proteins facilitate short-distance cell–cell communication. This activates direct signalling pathways that affect the cellular cytoskeleton. There is evidence which shows that Eph receptors can regulate actin dynamics via small GTPases of the Rho family (Rho, Rac and Cdc42) and therefore control cell shape by promoting the formation of stress fibres (Rho).



(A) Sc on Fc x30



(B) Sc on Ephrin x30

Figure 3.9: Representatives images of Schwann Cells on Fc coating (A) and EphrinB2 ligand (B). Plating cells on ephrinB2 results in a dramatic change in their morphology. When plated on Fc coating (A and C) Schwann cells have a more rounded shape, whereas on ephrin they become more elongated and form stress fibres.

N-cadherin is an essential component of adhesion junctions, which allow cells to form cell-cell adhesions. To understand whether cell-cell adhesion was required for Schwann cell morphology change, N-cadherin knockdown and control knockdown (kd) cells were cultured on plates coated with either clustered Fc or Fc-clustered ephrinB2 ligand.

Cells were allowed to attach and incubated for at least 24hrs. N-cadherin kd cells could not form clusters and did show not the elongated morphology typically seen in the cell clusters (see Fig. 3.10). However, in LCI experiments I observed that Schwann cells could form the elongated or polarised morphology without forming cell-cell contacts with other cells.

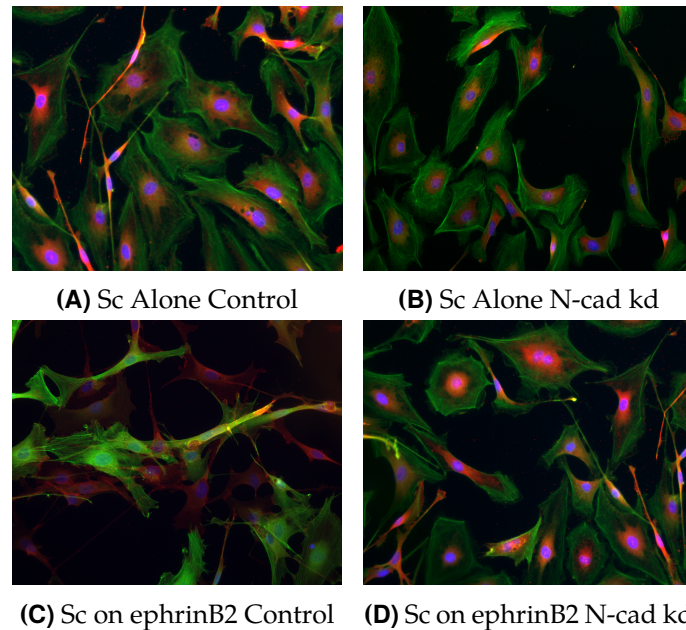


Figure 3.10: N-cadherin knock-down (B,D) and control knockdown (A,C) cells were cultured on plates coated with either clustered Fc or Fc-clustered ephrinB2 ligand. N-cadherin kd cells could not form clusters and did not the elongated morphology typically seen in the cell clusters when cells were plated on ephrinB2.

Impact of Fibroblasts on Schwann Cell Morphology in Migration Assay

To quantify the effect of fibroblasts on Schwann cell morphology in the migration assay, labelled (with CellTracker - a cell body dye) and unlabelled Schwann cells were mixed to create a population of sparsely labelled cells. This was necessary since when the Schwann cells are migrating in the presence of fibroblasts, they become tightly clustered and difficult to individuate. For quantitative analysis of cell orientation and elongation in the migration assay, Schwann cells were left to migrate either alone or with fibroblasts for 24-48hrs. Cells were then fixed and stained the cells for N-cadherin and labelled with DAPI. Fluorescent images were subsequently taken, and each picture was analysed using MATLAB (see Fig.refmigmorph). 15 images with 1-5 cells per image were examined to identify cell morphology. The ratio of the major to minor axes - the longest and the shortest length of the cell, respectively - were measured. This ratio was then subtracted by 1 to demonstrate a deviation of cellular shape from a perfect circle. The orientation angle of a polarized cell was determined by measuring the acute angle between the line of the major axis of the cell and the direction of average motion. A total of 50 cells were used to measure the polarisation angle distributions. An angle of 0 degrees would indicate that cells were perfectly aligned parallel to the mean direction of motion.

Schwann cells alone had a significantly larger minor axis than Schwann cells with fibroblasts (3.12B, Wilcoxon rank sum test, $p < 0.005$, $\alpha = 0.05$). A ratio of minor to major axis value of 0 would indicate a perfect circle and a value of 1 would indicate a line. Schwann cells migrating alone were more rounded than Schwann cells migrating in the presence of fibroblasts, (Wilcoxon rank sum test, $\alpha = 0.05$). Schwann cells alone were aligned perpendicular to the mean direction of motion ($\langle \theta \rangle = 89.7$ deg), while Schwann cells with fibroblasts were aligned to the direction of motion ($\langle \theta \rangle = 10.4$ deg), (Wilcoxon rank sum test, $p < 0.005$, $\alpha = 0.05$) as shown in Fig. 3.12C.

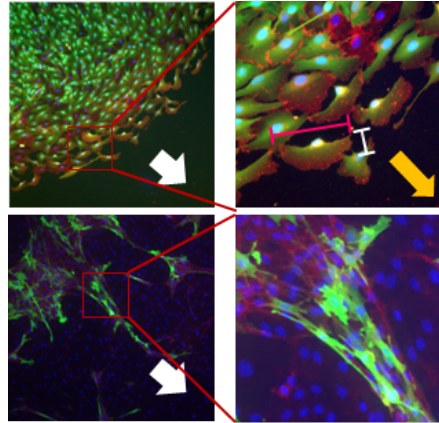


Figure 3.11: Schwann cells migrate alone (top) or with fibroblasts (bottom) in the migration assay. When alone, Schwann cells assume the classical fan shaped migration morphology, but when with fibroblasts they become elongated in the direction of motion. The direction of motion is showed by the white arrows.

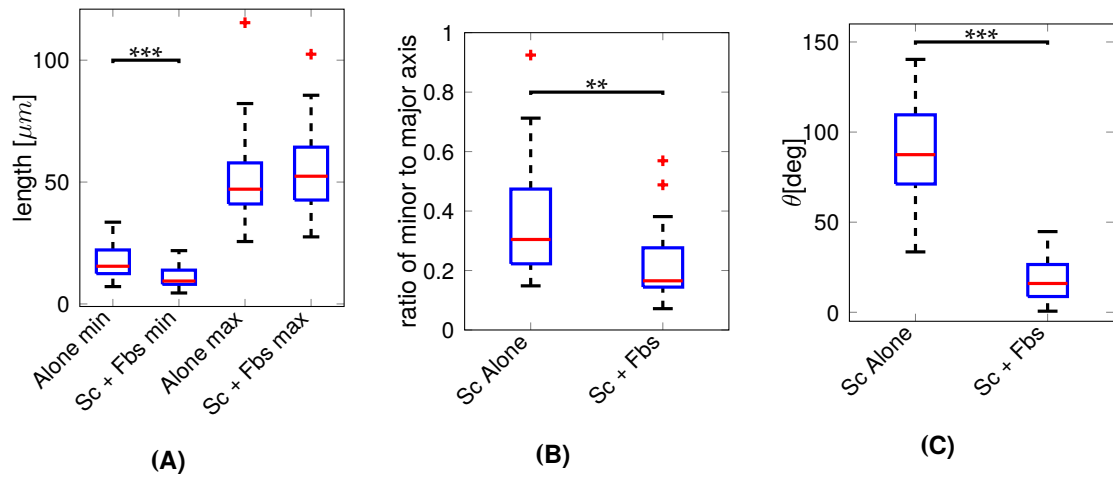


Figure 3.12: (A) Measurements of the minor and major axes of cells. Schwann cells alone have a significantly larger minor axis than Schwann cells with fibroblasts. (B) The ratio of minor to major axis, a value of 0 would indicate a perfect circle. Schwann cells migrating alone are more rounded than Schwann cells migrating in the presence of fibroblasts. (C) The alignment of the major axis relative to the outward mean direction of cell motion. Schwann cells alone are aligned perpendicular to the mean direction of motion ($\langle \theta \rangle = 89.7$ deg), whilst Schwann cells with fibroblasts are aligned to the direction of motion ($\langle \theta \rangle = 10.4$ deg). Wilcoxon rank sum test, $p < 0.005$, $\alpha = 0.05$ for all statistics.

3.4 Effect of fibroblasts on Schwann cell proliferation

A higher rate of cell proliferation at the leading edge of a group of cells can lead to directed cell colonization. It is known that following peripheral nerve injury there is a significant increase in Schwann cell proliferation (Fex Svennigsen A 2013). It has also been shown that restricting Schwann cell proliferation can disrupt successful nerve repair (Fex Svennigsen and Dahlin 2013). To determine whether fibroblasts impact Schwann cell proliferation *in vitro* Click-iT EdU technology (a proliferation assay) was used to measure the rate of new DNA synthesis in Schwann cells, which is an accurate method to measure proliferation in cell populations.

Schwann cells were labelled with cell-mask (for identification), and all cells were counter-stained with DAPI, to facilitate cell counting. Schwann cell proliferation was measured in Schwann cells cultured alone and with fibroblasts in both the co-culture assay and in the migration assay. In the Schwann cell/fibroblast co-culture assay, proliferating Schwann cells were identified by cells labelled with both EdU and cell mask. Cells were counted using a custom written code in MATLAB. Schwann cell proliferation is reported as the ratio of EdU-positive cells to the total number of Schwann cells. In the Schwann cell/fibroblast co-culture assay, proliferating Schwann cells were identified by cells labelled with both EdU and cell mask (see Fig. 3.13).

It is known that cell density can have an impact on cell proliferation rate via contact inhibition of proliferation (Fisher and Yeh 1967), therefore the rate of proliferation was measured both at an early and late time point, to create low and high cell density cases respectively (verified by cell count). I found that in both the high and low-density cases, Schwann cell proliferation in cells co-cultured with fibroblasts appeared to decrease (shown in Fig. 3.14), relative to Schwann cells in monoculture. However, Schwann cell proliferation in the high-density mono-culture was also lower than Schwann cell pro-

liferation in the low-density mono-culture. Furthermore, Schwann cell proliferation in the high-density co-culture was also significantly lower than Schwann cell proliferation in the low-density co-culture (shown in Fig. 3.14). This suggested the decrease in Schwann cell proliferation from co-culturing with fibroblasts could be explained by the increase in cell density.

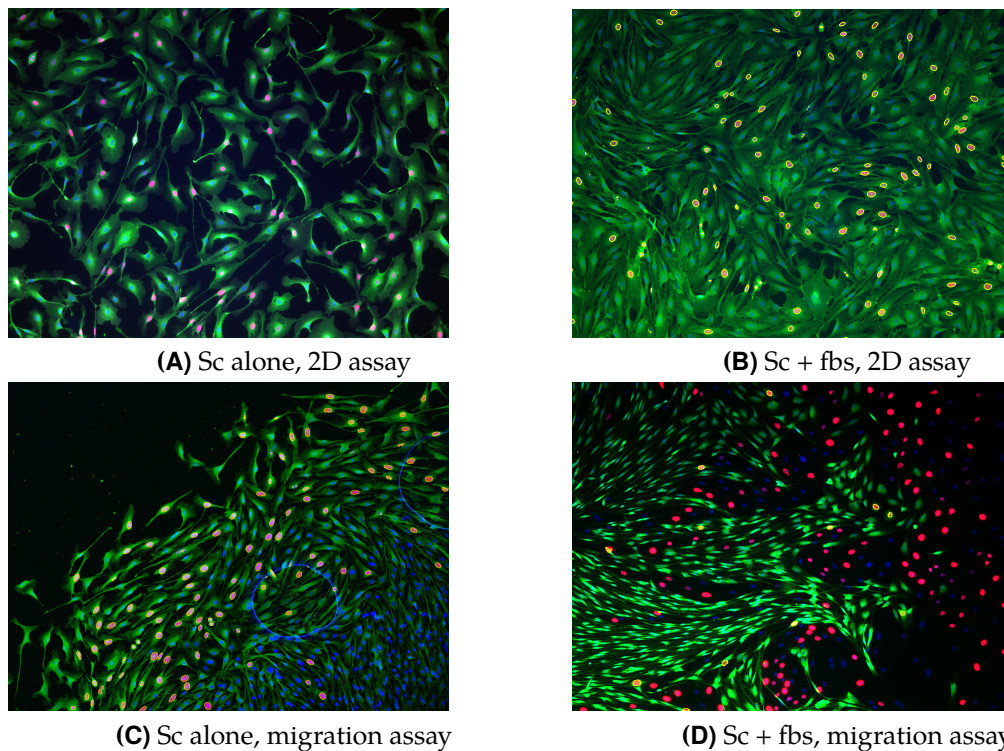


Figure 3.13: Schwann Cell Proliferation: Representative images of data from proliferation assay experiments. Schwann cells are labelled in green (Cell Tracker: cell body label). Cells which have divided within a 6h period are labelled with EdU (Red), and all cells are labelled with Dapi (nuclear marker: Blue). Schwann cells are either alone (A and C) or with fibroblasts (B and D), in the co-culture assay (top) or migration assay (bottom). In both assays, the presence of fibroblasts appears to decrease Schwann cell proliferation, however normalising using the total (Schwann cell plus fibroblasts) cell density suggests this effect is a result of increased cell density. Note, Schwann cell proliferation is labelled with a yellow outline. All cells (fibroblasts and Schwann cells) are labelled with EdU, therefore to identify Schwann cells which have proliferated, only nuclei which are labelled with both cell-mask and EdU are counted.)

The experiments showed that co-culturing Schwann cells with fibroblasts had no stimulating effect on the proliferation of Schwann cells. Rather it appeared the rate of proliferation decreases. However, I suggest that this effect is a result of increased cell density rather than from active signalling (McClatchey and Yap 2012; Gérard and Goldbeter 2014). These results mean I did not need to implement a proliferation term into the computational model which I used to simulate collective cell migration. They also show, that although increased Schwann cell proliferation is important for successful nerve repair, it does not have a role (here) in the collective cell migration phenomenon.

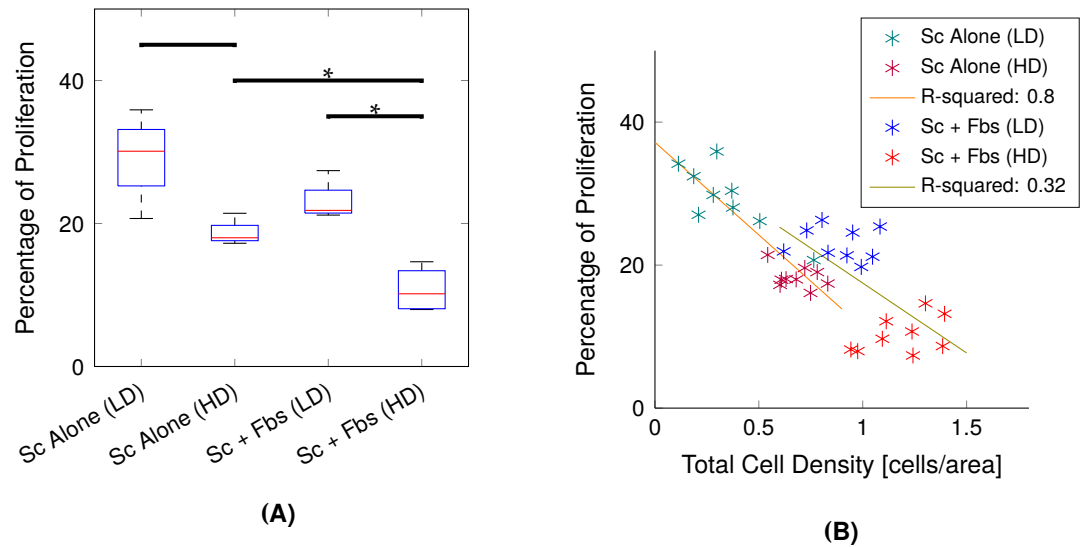


Figure 3.14: Quantifying Schwann cell proliferation. (A,B) Schwann cell proliferation in Schwann cells co-cultured with fibroblasts appeared to decrease compared to the proliferation rate of Schwann cells in a monoculture. However, this can be explained by the increase in cell density. Co-culturing Schwann cells with fibroblasts has no stimulating effect on the proliferation of Schwann cells. Rather, it appears the rate of proliferation decreases, however, this effect is likely due to the increase in cell density rather than from active signalling. This is demonstrated by the decrease in cell proliferation in both the mono-culture (Sc alone) and the co-culture (Sc + fbs) in the high cell density (HD) experiments compared to the low cell density (LD). Statistics: Wilcoxon rank sum test is used ($p < 0.05$).

3.5 Effect of fibroblasts on clustering in Schwann Cells

Work by Parrinello et al., showed in a qualitative way that fibroblasts can induce clustering or sorting behaviour in Schwann cells (Parrinello 2010). Cell sorting is the process by which two or more mixed populations of cells segregate themselves into their distinct populations. This results in clusters of Schwann cells being formed, typically with 5-10 cells per cluster. To investigate whether ephrinB2 could reproduce the clustering effect I compared fibroblast induced cell-sorting to ephrinB2 induced clustering. Schwann cells (labelled with cell mask) were seeded either on their own, on Fc-clustered recombinant ephrinB2, or with an equal number of fibroblasts and after 24h the spatial distribution of the Schwann cells was analysed.

When Schwann cells were plated onto tissue culture plates, they adhered to the surface without any intrinsic spatial pattern. Therefore, it can be assumed that any significant departure from a random spatial distribution is due to effects from interaction with the fibroblasts. To quantify this I used point pattern analysis. Point pattern analysis (PPA) is the study of the spatial arrangements of points in space. PPA can be used to infer whether a given arrangement of points in space is random, uniform or clustered. To extract the spatial position of the cells, I used Dapi (nuclear fluorescent stain) to label the cell nuclei. Then, using image processing methods of thresholding, morphological operations and region identification, I extracted the x-y coordinates of the cells (see Methods for details) - Figure 3.15. This is a relatively easy task when Schwann cells are alone, however, when the Schwann cells are induced to cluster together, overlapping nuclei make this a more challenging task.

The homogeneous Poisson point process (equation 3.3) serves as a reference model for a completely spatially random (CSR) point pattern (Schabenberger and Gotway 2005). To measure spatial patterns a distance method (equation 3.5), comparing the empirical

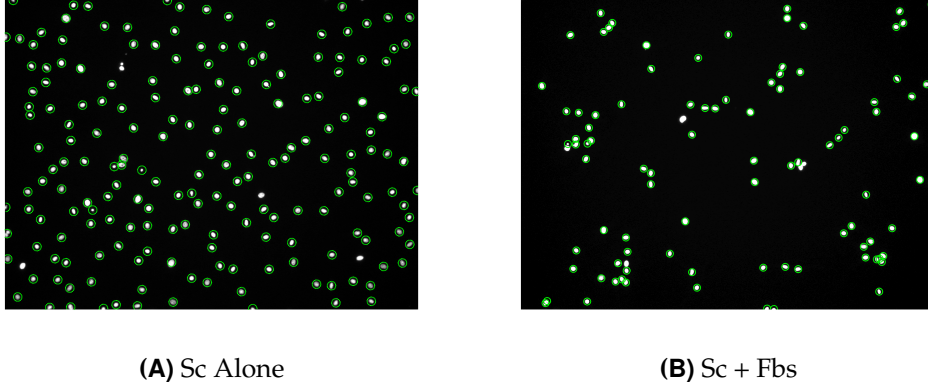


Figure 3.15: Dapi images of Schwann cell nuclei when cultured alone (A) or co-cultured with fibroblasts (B). When Schwann cells are clustered alone (A), Schwann cells appear uniformly distributed, spread out evenly over the available space. Fibroblasts induce a clustering phenotype. The Sc in (B) form tight local clusters of roughly 5-8 cells.

distribution function (EDF) of distances between events with an EDF that is based on the CSR assumption (equation 3.4).

$$P(N(A) = n) = \frac{e^{-\lambda\nu(A)} [\lambda\nu(A)]^n}{n!} \quad (3.3)$$

where $N(A)$ is the number of events in A (area) and λ is density. The distribution function as used in (Schabenberger and Gotway 2005) for the nearest neighbour distance for CSR is given by:

$$G(r) = 1 - e^{(-\lambda\pi r^2)}, r \geq 0 \quad (3.4)$$

Nearest neighbour analysis examines the distance between each point and the closest point to it, and then compares these to the expected values for a random sample of points from a CSR.

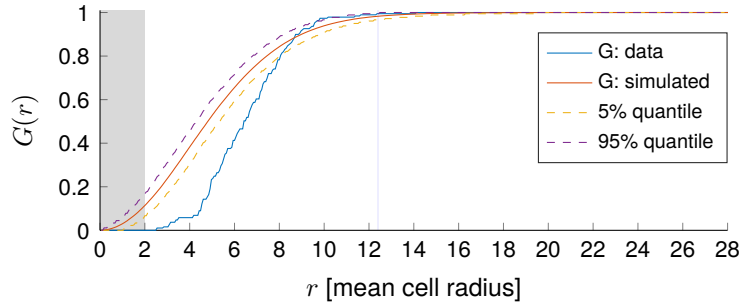
$$\hat{G}(r) = \frac{1}{n} \#(i : r_i \leq r) \quad (3.5)$$

Where r_i is the distance from x_i to the nearest point of A, and $\#$ denotes the number of events in the set. The empirical nearest neighbour function $\hat{G}(r)$ gives the probability

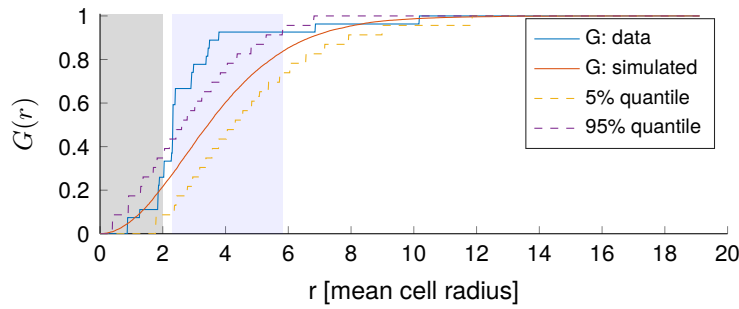
of an observed nearest neighbour of a point appearing at any given distance r . It describes the degree of clustering or regularity in the point pattern. Values of $\hat{G}(r)$ greater than for a data set under CSR imply the data set is a clustered, smaller values of $\hat{G}(r)$ describes a more uniform pattern.

The empirical distribution function of experimental data was calculated using a custom written code I wrote in MATLAB based on the equation in 3.5. I ran 2000 independent simulation of CSR and by computing the estimated G function for each of these, set upper and lower envelope of these simulation curves (95% percentile). If the summary (G) functions obtained from the observed point process depart does not lie within the simulation envelope, then there is sufficient evidence to reject the null hypothesis for CSR (see Fig. 3.16). I compared the spatial distribution of Schwann cells to those plated with fibroblasts, and compared both these scenarios to the mean simulated CSR distribution n , using the envelope method described above to identify a significant departure from CSR. This method provides an unbiased measure of clustering.

To compare between different experimental repeats or to evaluate effects of perturbing the system I use three methods to measure the difference of empirical data distribution to CSR; the maximum distance to CSR, the area between CSR and empirical distribution. The resulting measures are signed, the sign encapsulates information of the type of pattern (i.e. more clustered or more uniform), and the magnitude reports the relative extent of the pattern. The drawback to this method is that it does not incorporate any morphological information about the cells. However, as a readout of clustering, it is an effective measure and one which allows for quick evaluation of large data sets. These results show that Schwann cells plated on a coating of clustered recombinant ephrinB2 formed clusters which had the same clustering read-out as Schwann cells co-cultured with fibroblasts (see Fig. 3.17). This shows that specifically ephrinB2/EphB2 signalling between Schwann cells and fibroblasts plays a key role in the cell clustering behaviour.



(A) Summary statistics of cell clustering when Schwann cells are cultured alone. Data is more uniform than random.



(B) Summary statistics of cell clustering when Schwann cells are co-cultured with fibroblasts. Data is more clustered than random.

Figure 3.16: Empirical data (blue) is compared to the average (orange) of 2000 simulations under CSR and identifies as significant when it falls outside the 5th (or 95th) percentile. When Sc are cultured alone, the CDF falls below the CSR, implying there is a tendency to uniformity in the spatial distribution of the data. When Sc are co-cultured with fbs, the CDF is above the CSR, showing clustering in the spatial distribution. The shaded grey region shows the area where the search radius is less than the mean diameter of a cell. The shaded blue region shows the region where the empirical data is significantly above the simulated CSR.

When Schwann cells were plated alone, the point pattern was typically more uniform than totally random. This is because the spatial pattern of the cells is analysed after at least 24hrs, by which time the number of cells has increased (proliferation), leading to an increase in cell density. Schwann cells arrange themselves into a regular uniform pattern in response to homotypic contact inhibition of locomotion.

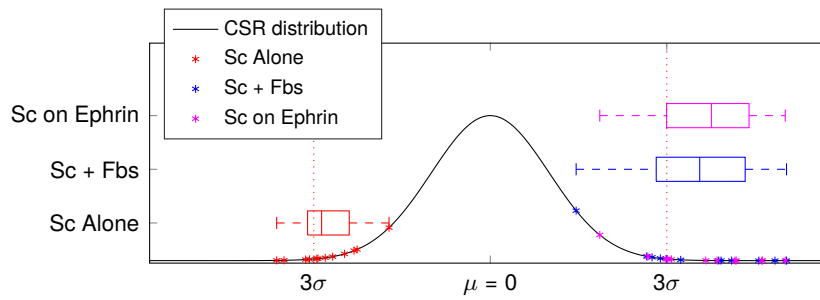


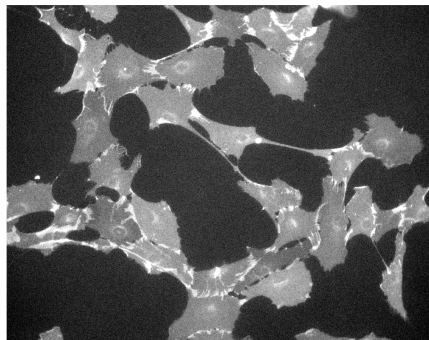
Figure 3.17: Cluster formation is measured using spatial statistics. We show the area difference between the cumulative distribution of distances from arbitrary event to nearest event and the complete spatial randomness (CSR) distribution. This value will be negative for spatial distribution which are tending to uniformity and positive for spatial distribution tending to clusters. Schwann cells co-cultured with fibroblasts or plated on EphrinB2 show high levels of spatial clustering, while Schwann cells plated alone show a more random to uniform spatial distribution.

3.6 Quantification of N-cadherin relocalisation

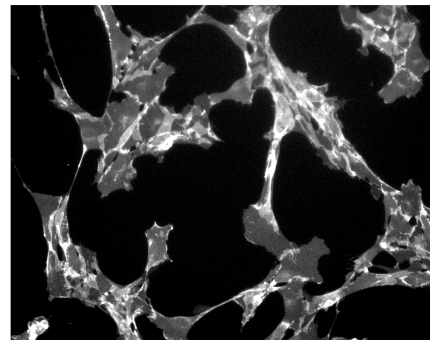
Work by (Parrinello 2010) showed that fibroblast-mediated cell sorting involves N-cadherin re-localisation to Schwann cell contacts. They also showed that endogenous levels of N-cadherin protein in the Schwann cells do not increase upon interaction with fibroblasts until after 24hrs when the clustering phenotype is already established. N-cadherin was detectable in Schwann cell monoculture, found throughout the cytoplasm and at the membrane. The spatial location pattern of N-cadherin dramatically changed in the Schwann cells in co-culture with fibroblast with a significant increase in levels at cell-cell contacts. In the previous section, I showed that ephrinB2 could induce the same clustering pattern observed when Schwann cells are co-cultured with fibroblasts and undergo a cell sorting process.

EphrinB2 coating had a similarly dramatic effect on the spatial location of N-cadherin. N-cadherin was relocated from the cell body to cell-cell contacts - see Fig Figure 3.18. Measuring this, a two fold increase in N-cadherin localised at the cell periphery in

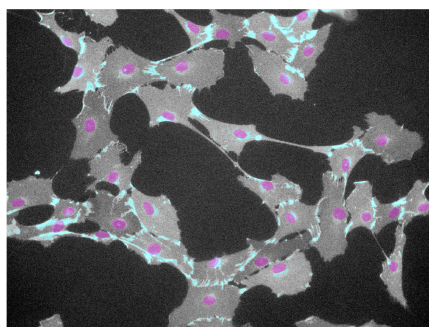
Schwann cells cultured on ephrinB2 to Schwann cells cultured alone was seen (see Fig. Figure 3.19). These results reinforce the idea that ephrinB2 plays the key role in N-cadherin relocalisation, and it is this process which is key in the formation of clusters of Schwann cells.



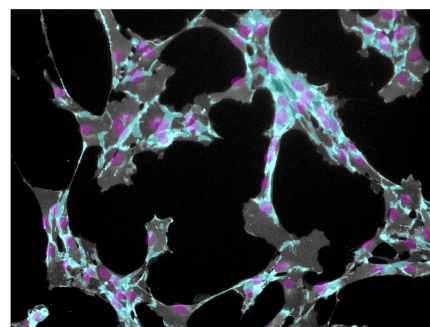
(A) Schwann Cells on Fc



(B) Schwann Cells on Ephrin



(C) Schwann Cells on Fc



(D) Schwann Cells on Ephrin

Figure 3.18: Schwann cells are plated either on Fc alone or on ephrinB2 coated plates clustered with Fc. In response to ephrinB2, Schwann cells form tight clusters (over the course of about 24h). Cells were labelled with Dapi and stained with N-cadherin antibody. Raw images of N-cadherin staining is shown in figures (A) and (B). Using image processing methods in MATLAB, I identified regions on N-cadherin located at the cell periphery (blue) and used this as readout of N-cadherin re-localisation. The number of cells was counted using cell nuclei (pink).

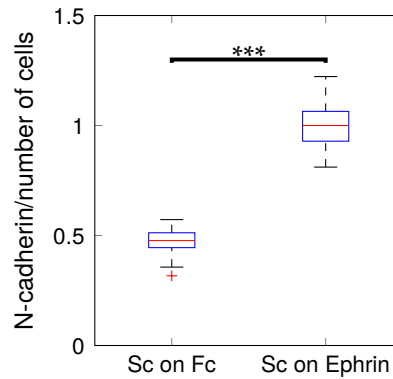


Figure 3.19: Box and whisker plot showing the relative amount of N-cadherin at cell periphery. Box: 25th and 75th percentiles, bars: minimum and maximum values, red crosses: outliers. Red line: median value. Values reported are the number of pixels which are at the cell periphery and which are labels with N-cadherin. The number of independent experiments is 7 (n = 7), with 4 biological repeats for each n (total images processed: 28). Results are normalised to the median value of Schwann cells on clustered ephrinB2 ligand. Line over boxes signify significant difference. Significance tested for using Welch's t-test with unequal variance- (p < 0.001).

To understand whether this effect was also present in a collective migration setting, Schwann cells were prepared in the migration assay and allowed to migrate out either into fibroblasts or alone for 48hrs. Cells were then stained for N-cadherin and labelled with Dapi (see Fig. Figure 3.20. N-cadherin levels at cell-cell contacts were much higher when Schwann cells are migrating surrounded by fibroblasts then when migrating alone, however N-cadherin levels at free cell edges (Fig. 3.20 bottom right), e.g. the "leader" cells at the tip of the cord. This is in agreement with the suggested concept of "leader-follower" cell, where strong adhesions act to connect the leader and follower cells, resulting in emergent collective features such as collective polarisation.

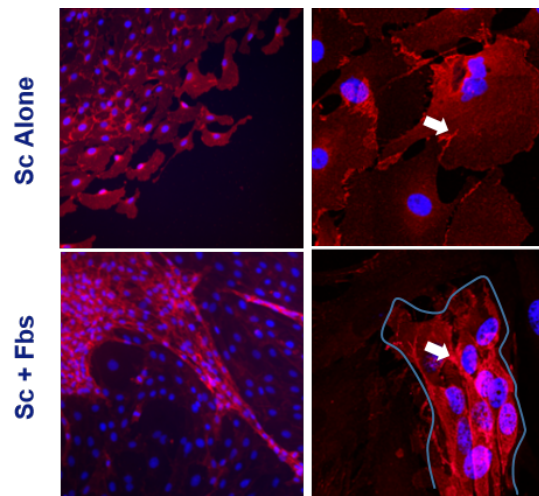


Figure 3.20: Localisation of N-cadherin in the migration assay. N-cadherin is re-localised to cell-cell contacts when fibroblasts are present, increasing the amount of N-cad at cell-cell adhesions compared to when no fibroblasts are present.

3.7 Fibroblast soluble factors stimulate Schwann cell migration

Schwann cells cultured with fibroblasts had a higher migration speed than Schwann cells cultured alone. To determine the effect of soluble cues derived from fibroblast on Schwann cells a Boyden chamber assay was used (Boyden 1962), see Fig. 3.21). Schwann cells were plated on the insert membrane, and different combinations of media were used in the upper and lower chambers: (1) culture medium plus 10% serum in (upper) and (lower) (negative control), (2) culture medium plus 2% serum in (upper) and plus 10% serum in (lower) (positive control). All other combinations had 2% serum in (upper) and (lower) in addition to media preconditioned for 48hrs by fibroblasts at densities 4×10^5 cells per well in the lower chamber. In (3) and (4) nerve fibroblasts were present throughout the assay. Results are shown for the low serum media, as these experiments are most representative of a pure fibroblast derived motility effect. The effect of the soluble factors derived from fibroblasts was measured by quantifying the number of cells which migrated through the insert

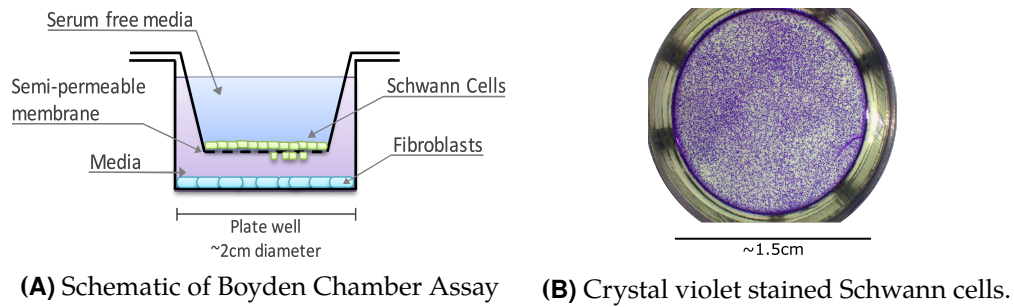


Figure 3.21: (A) A schematic of the Boyden chamber experiment. Schwann cells are plated onto a trans-well insert. The base of the insert is a semi-permeable membrane, which the cells can migrate through if they are attracted by some chemoattractant on the other side. (B) Representative image of the underside of the insert after staining with crystal violet.

membrane. Schwann cells were removed from the top of the insert, fixed in 4% PFA and stained with crystal violet. Representative images 6 per insert were taken and analysed in MATLAB (for examples see Fig 3.22A and 3.22C). The readout of motility is the averaged percentage area occupied by cells. The area occupied was found by thresholding the image to select only areas labelled with crystal violet (see Fig 3.22B and 3.22D for examples of thresholded images overlayed on raw images). Fibroblasts cultured in the well below the inset and conditioned media increased Schwann cell motility up to 10 fold (see Fig 3.23). This is in agreement with the results presented showing that the speed of Schwann cell motion was significantly higher when Schwann cells were in co-culture with fibroblasts than when in a monoculture.

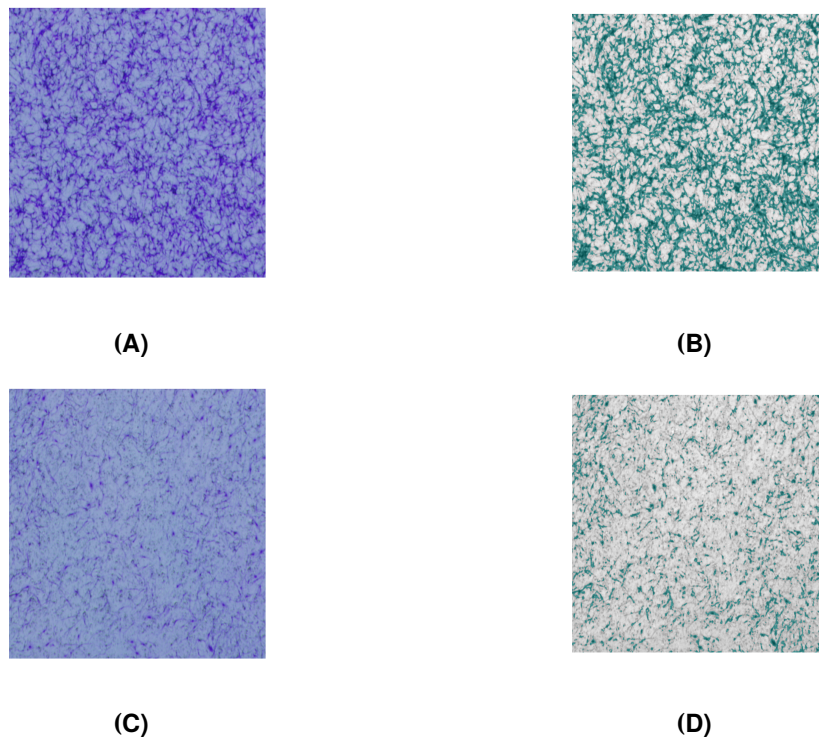


Figure 3.22: Examples of data from Boyden Chamber experiments. Schwann cells on an insert above fibroblast conditioned media (A) and above serum-free (C). (A, C) raw images of the Boyden chamber inserts, (B, D) thresholded image false coloured in teal.

3.8 Summary and Conclusion

To understand the effect of fibroblasts and ephrinB2 on Schwann cells cell I looked at cell clustering, localisation of N-cadherin, cell morphology, speed, motility, and persistence in motion. Co-culturing Schwann cells with fibroblasts had clear effects on the morphology, motility and mode of migration of Schwann cells. Schwann cells had a higher speed of migration and tended to adopt an elongated morphology, which defined the direction in which they move. Recent studies have shown that a unipolar or asymmetric bipolar phenotype is characteristic of motile and migratory Schwann cells (Mitchel and Hoffman-Kim 2011), while symmetric bipolar and multipolar mor-

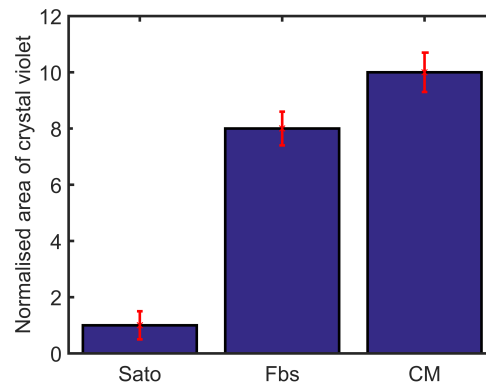


Figure 3.23: Results of Boyden Chamber Assay. Cell migration through a permeable membrane is measured by quantifying the area on the underside of the membrane occupied by cells. The area occupied by cells was significantly higher when fbs were cultured in the bottom well or when conditioned media (from fbs) was used in the bottom well, compared to when Sato (serum free media was used).

phology is associated with less motile phenotypes, and our results are in agreement with these findings. Higher motility and shape change was correlated with greater directionality, however, an interaction between a Schwann cells and fibroblast resulted in a re-polarisation of the Schwann cell away from the point of interaction, interrupting directional motion. These results suggest that fibroblast have a significant impact on Schwann cells concerning cell motility and morphology (which are often inherently linked).

Schwann cells in a monoculture do not form clusters and typically adopted either a more rounded shape or fan shaped migratory morphology. In the monoculture, contact inhibition between cells explained the random or uniform (depending on cell density) spatial arrangement of cells. Schwann cells in a monoculture are motile, and their migration is random with little persistence.

Schwann cells in co-culture with fibroblasts or cultured on ephrinB2 formed clusters. This was quantified using point pattern analysis and spatial statistics. The level of

clustering in Schwann cells in co-culture with fibroblasts and Schwann cells cultured on a coating of clustered recombinant ephrinB2 was of the same degree, suggesting that ephrinB2 signalling is sufficient to induce clustering.

In the presence of fibroblasts, via ephrinB2 signalling, N-cadherin (usually found dispersed through the cell or in small amounts at cell-cell contacts) was relocated to cell-cell contacts. The re-localisation of N-cadherin to cell-cell contacts both in Schwann cells co-cultured with fibroblast and cultured on ephrinB2 suggested N-cadherin plays a key role in the clustering process. Quantitatively this showed a two-fold increase in N-cadherin located at cell-cell contacts. Temporarily removing N-cadherin via siRNA knock-down prevented clusters from forming. This, combined with a reduction in the CIL between Schwann cells results in a cell sorting process in the co-culture assay. In the migration assay, this effect of N-cadherin re-localisation may play a role in the directional polarisation of the Schwann cells.

In addition to the formation of clusters, Schwann cells in co-culture with fibroblasts or cultured on clustered ephrinB2 underwent a clear change in morphology. Although different morphologies were observed, the key features were that Schwann cells became elongated and bipolar, exhibiting a phenotype typically associated with a higher motility. This morphology change was also observed in the migration assay, which implied that this is also a relevant feature in the collective cell migration setting. The migration speed of Schwann cells in co-culture also increased. Soluble factors from fibroblasts increased motility or invasiveness of Schwann cells, as shown in the Boyden chamber assay.

From measuring the rate of DNA synthesis with EdU showed that the rate of proliferation is not sufficient to result in the migratory phenotype that observed in the migration assay. Results showed fibroblasts reduced the rate of proliferation, however, density

dependent experiments showed that this is likely due to an increase in cell-density and therefore reduction in free space. Concerning the migration assay, from these results, I believe Schwann cells migrating outwards due to anisotropic topography via interactions with the substrate or anisotropic cadherin-mediated cell–cell adhesion rather than directional proliferation.

Table 3.1: Cell Features

Feature	Sc Alone	Sc + fbs
Spatial Organisation	Random	Clustered
N-cadherin	Cell Body	Concentrated at cell-cell contacts
Morphology	Rounded/flat/fan	Elongated/polarised
Motility	Motile	more motile
Persistence	No persistent motion	Persistent motion when polarised

Collective migration is more than the sum of its parts. Although these results clarify the effects of fibroblasts on Schwann cells, they do not explain how these features play a role in collective Schwann cell migration, nor how cell-cell contacts modify these features. Most collectively migrating cells are homotypic, i.e. from one population of cells, collective motion can arise (Chapnick and Liu 2014; Yamaguchi et al. 2015; Yang et al. 2016). In this system, fibroblasts have such a clear effect on the change of cell-cell adhesion that it seems that fibroblasts play a key role in inducing a collective migration phenotype in Schwann cells. However, the impact of fibroblasts on Schwann cell morphology, motility and persistence also appear to be important features which we should consider in the collective setting.

4

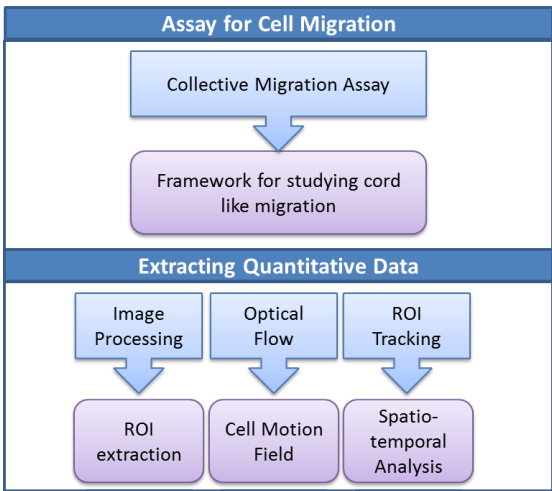
Collective Cell Migration

4.1 Introduction

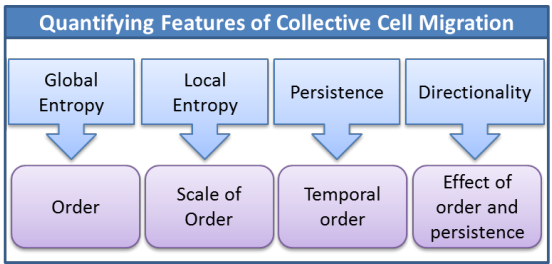
In the first part of this chapter, I describe the migration assay I developed and optimised which allowed me to recapitulate the cord-like migration observed in PNS repair *in vivo* and thus the framework to study the cord like collective cell migration of Schwann cells. I then describe the methods and validation for image segmentation, extracting cell velocity fields by calculating the optic flow, and methods for tracking groups of cells using optic flow. Using image processing, I can segment phase contrast images to identify and label regions of Schwann cells, empty regions and regions of fibroblasts,

which minimise noise. The application of optic flow and phase contrast segmentation provides a way to extract quantitative data from live cell imaging of collective migration. Finally, I use optic flow to extract velocity fields from the live cell imaging data I show that this method can be used to measure cell movement. I also show that the optic flow can be used to track a region of interest to facilitate tracking groups of cells and time series analysis of behaviour with respect to spatial location such as at the leading edge (leader cells) compared to follower cells.

In the second part of this chapter, I describe the analysis and metrics applied to the quantitative data. I want to measure features of behaviour which will (a) help to clarify the impact of fibroblasts on Schwann cell migration and (b) can be used for parametrization and model comparison. For this reason, I use four key metrics, which capture different pieces of information. I use global entropy as a measure of global order, local entropy as a measure of local coherence (how similar the direction of motion is between neighbouring cells). To quantify motion and order of motion over time, I use persistence and measures of directionality.



(A) Part I



(B) Part II

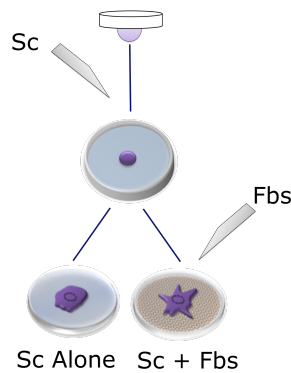
Figure 4.1: (A) Part 1 of this chapter covers the migration assay used and the methods for extracting relevant quantitative data. (B) Analysis methods and results using the quantitative data.

4.2 Collective Migration Assay

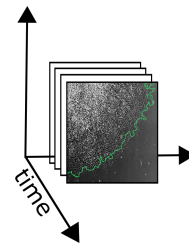
One of the key features I am interested in is the emergence and persistence of cord-like structures. The typical assay studied in collective migration has been the wound healing assay which is an easy and inexpensive method to study directional cell migration *in vitro* (Liang, Park, and Guan 2007). A “wound” is created in a cell monolayer by scratching the monolayer with a needle; images are recorded at regular intervals during cell migration. It can be used to study the effects of cell type or pharmacological intervention on cell migration. As the field of collective cell migration is growing, a number

of other migration/invasion assays are available, which are reviewed in (Kramer et al. 2013).

Since I was interested in how fibroblasts can change the behaviour of cord like Schwann cell migration I adapted and optimised an assay which allowed me to recapitulate the cord like protrusions seen *in vivo*. I will refer to this assay as the "migration assay" within the text of this thesis. Schwann cells are aggregated using a "hanging drop assay". Once the cells have formed bonds (usually after 6-8hrs), small pieces are separated from the main clump using a pipette and plated onto tissue culture plate in a 0.5% matrigel solution. After the Schwann cells have attached, fibroblasts are then added to the plate, and after these have attached, the migration is recorded in a live cell imaging set up (see Fig. 4.2). This assay can recapitulate the cord like collective migration which is observed *in vivo*.



(A) Migration Assay Setup



(B) Recording Collective Migration

Figure 4.2: (A) Schwann cells aggregate in a hanging drop assay. Once the cells have formed bonds, a small clump is separated off and placed in a well of a 12 well (PLL coated) tissue culture plate in a 0.5% matrigel solution. (B) After these Schwann cells have attached, fibroblasts are then added to the plate, and after these have attached, the migration is recorded in a live cell imaging set up. Images are recorded every 10 minutes for up to 72hrs.

In this assay, due to the high density of Schwann cells, the cells migrate out from the aggregate. When there are no fibroblasts, cells migrate in a loose sheet. Transient bonds form between cells, especially in the early time points since cells are forced to be in contact due to lack of space. However, these are not maintained (for representative image see figure 4.3A). Although there is an overall general motion outwards, there is little cohesive movement between neighbouring cells; individual cells often move perpendicular to the overall outward direction of motion. Cells at the leading edge (i.e. those with most surrounding free space) often break away from the main group of cells, and in these cells, the outward motion observed in the group is lost, and the cells undergo a more random migration. At later time points, the outward motion is damped, and cells fill out space without forming lasting contacts or forming clusters.

When fibroblasts are present, Schwann cell migration behaviour is notably different. Schwann cells migrate out in tight cord like formations (see figure 4.3B). Schwann cells in the cord are densely packed, and the cords last for long periods of time, although they exhibit dynamic behaviour and can reorganise and split into “sub-cords” while maintaining the bonds between Schwann cells. The collective cord maintains persistent motion, however also appears to steer around or be deflected away (depending on the density of cells in the cord) from fibroblasts. The cords typically form in the outward direction of motion. Directional changes occur at the tip of the cord, where cells search for free space or interact with fibroblasts.

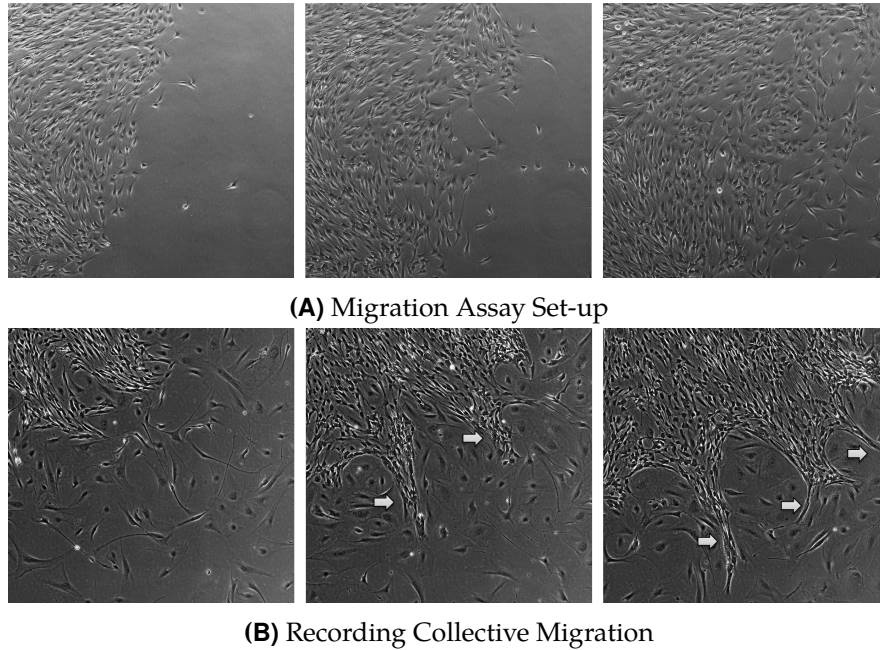


Figure 4.3: Snap shots of the migration assay at different time-points (left to right $t = 0$, $t = 4\text{hrs}$, $t = 8\text{hrs}$) for (A) Schwann cells alone. As time progresses (left to right) Schwann cells migrate out, spreading out but forming no clear structures. (B) Schwann cells with fibroblasts. As time progresses (left to right) Schwann cells migrate out and start to form cord-like structures. White arrows (B, middle and right) indicate where cords have formed. These cords recapitulate (to some extent) the cord like structures of Schwann cells which are essential in peripheral nerve repair *in vivo*. This assay allowed me to study the migration of Schwann cells in these cord like structures, and how they differ from Schwann cells when migrating in the loose (non-collective) mode as shown in (A).

Image Processing: Masking Phase Contrast Images

It is necessary to be able to distinguish between the Schwann cells and fibroblasts. To identify a population of cells, cells can be labelled with a fluorescent marker, and data recorded using white light and light at the excitation frequency of the marker. During live cell imaging, to capture a large enough region of interest, we record multiple images for a given area and stitch them together to form a tiled image. This means the cells are exposed to a large amount of stress from prolonged fluorescence imaging which can lead to cell death or unexpected behaviour. To avoid this issue, I used the properties of

phase contrast microscopy, which -combined with suitable image processing methods- means I could identify regions of Schwann cells and fibroblasts) thus avoiding this issue and the necessity for labelling and fluorescent imaging of cells.

To segment cells, the contrast of the original image is enhanced by transforming the values using contrast-limited adaptive histogram equalization (Fig. 4.4A). The image is then converted to a binary image (Fig. 4.4B), and the background is removed by performing morphological opening operations on the binary image with a disk structuring element with a radius parameter p_1 which is optimised for each image set (Fig.4.4C). Holes in the binary image are filled, and morphological closing (dilation followed by erosion, using the same structuring element for both operations) is performed on the binary image using a disk structuring element with a radius parameter p_1 which is optimised for each image set (Fig.4.4C). Finally, an operation to remove all connected components that have fewer than p_3 pixels is applied to the binary image (Fig.4.4E). This final image is used to mask the raw image and with simple binary operations can be used to select only Schwann cells, only fibroblasts or find the boundary between the two cell types (e.g., Fig.4.4F).

For each set of images (video recordings), the parameters used to perform the image processing operations were optimised by a grid-search of suitable parameters (determined initially by trial and error) and assessing by eye (overlaying the mask on the phase contrast image) how well the mask did. The range of optimal parameters was very different in the images where there were fibroblasts compared to when there were none, however within each category the parameters were very similar. Furthermore, since the distribution of image intensity is very stable over the recording time (i.e., the image intensity stays the same), the parameters for one frame could be used for all frames (see fig. 4.5). This is another advantage over creating masks from fluorescent images since the signal variation in fluorescent images is much greater than in phase

contrast images.

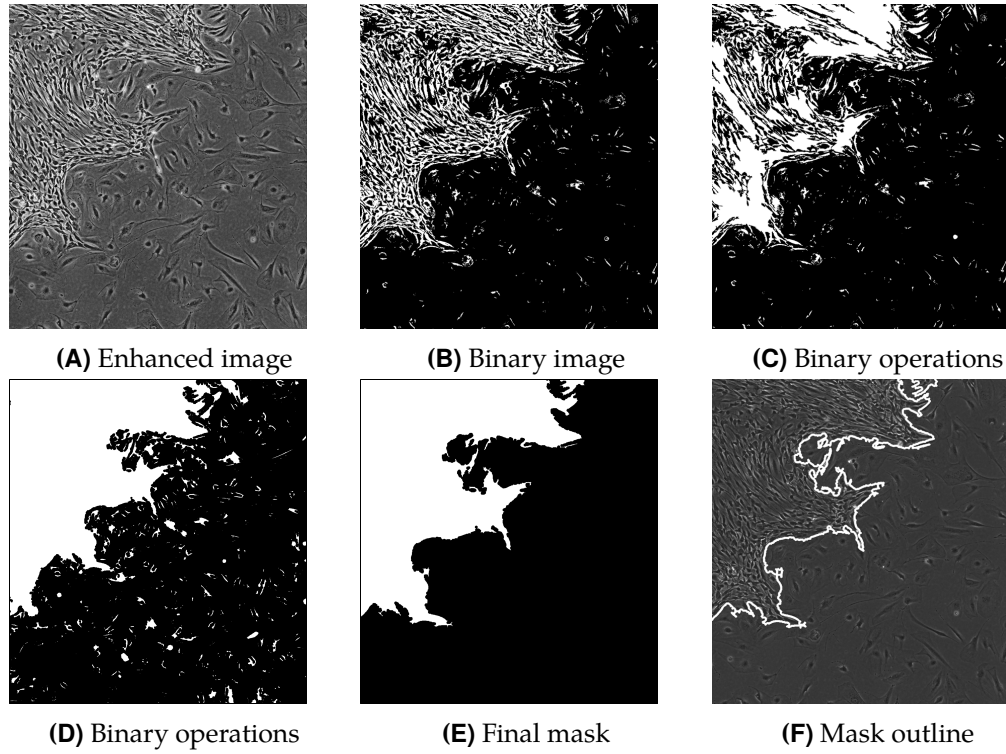


Figure 4.4: Example images in the process of segmenting phase contrast images. Images are enhanced, the background is removed and a binary image is created. Binary operations, filling, eroding and dilating are performed to create a mask of Schwann cells only. This mask can be manipulated to show Schwann cells only, fibroblasts only, or show the line between them.

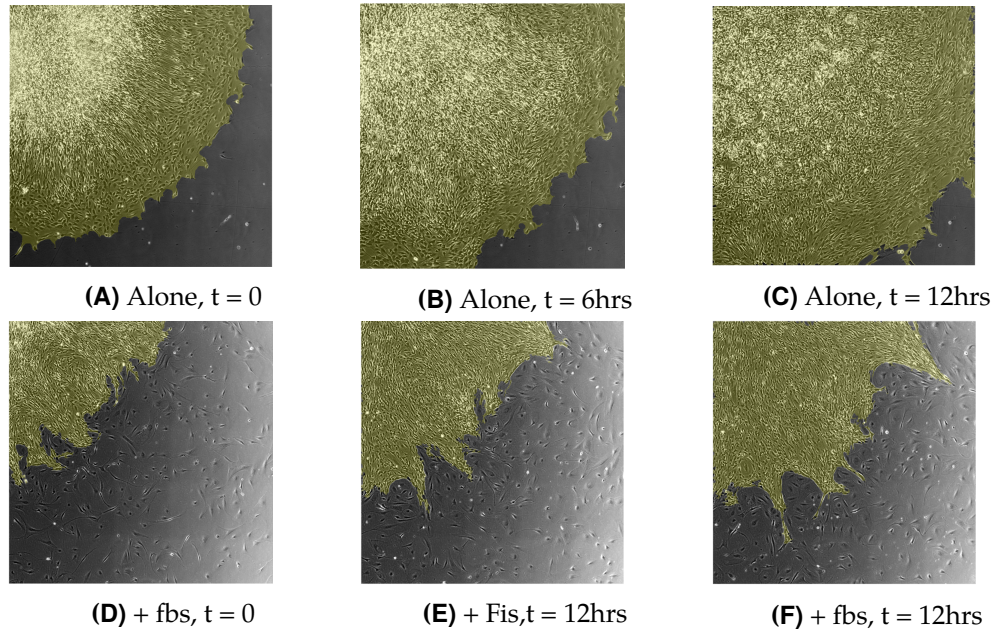


Figure 4.5: Showing masks created from the original bright field images. Frames from three time points are shown with their corresponding mask overlayed to indicate Schwann cells.

To verify that this method can be used to accurately differentiate between different cell types/between cells and background I compared phase contrast generated masks to masks extracted from segmentation of the fluorescent image. Using the method described above a mask from the phase contrast image was created. From the fluorescent image, noise/background was removed, and a binary image was created. To compare the two masks a pixel by pixel difference was calculated and the two masks were compared to the phase contrast image (see Fig.4.7).

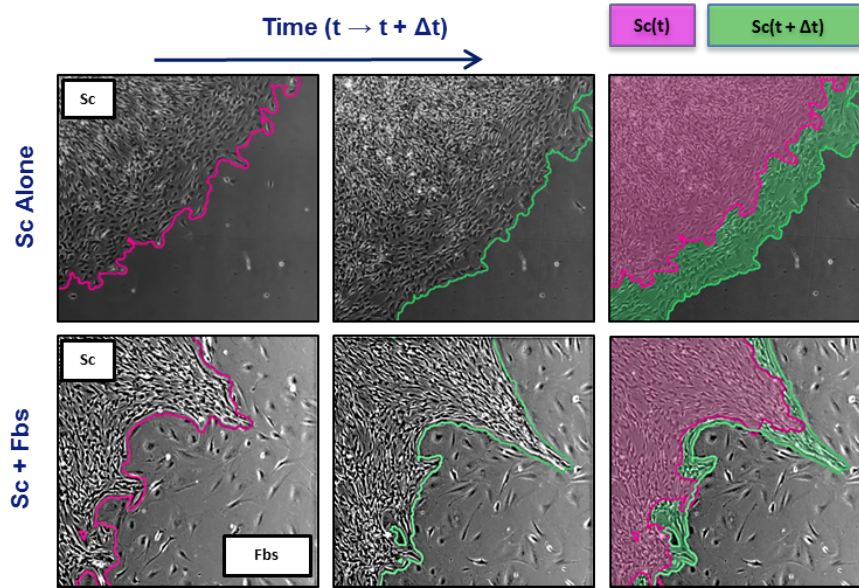


Figure 4.6: Live cell imaging with applied masks shows the progression of Schwann cell migration. The pink line (left) shows the migration from at time t , and the (middle) green line shows the position of the migration front 1hr later. The area of Schwann cells is shown (right) by areas in pink (cells at time t), and green (cells at time $t + 1hr.$)

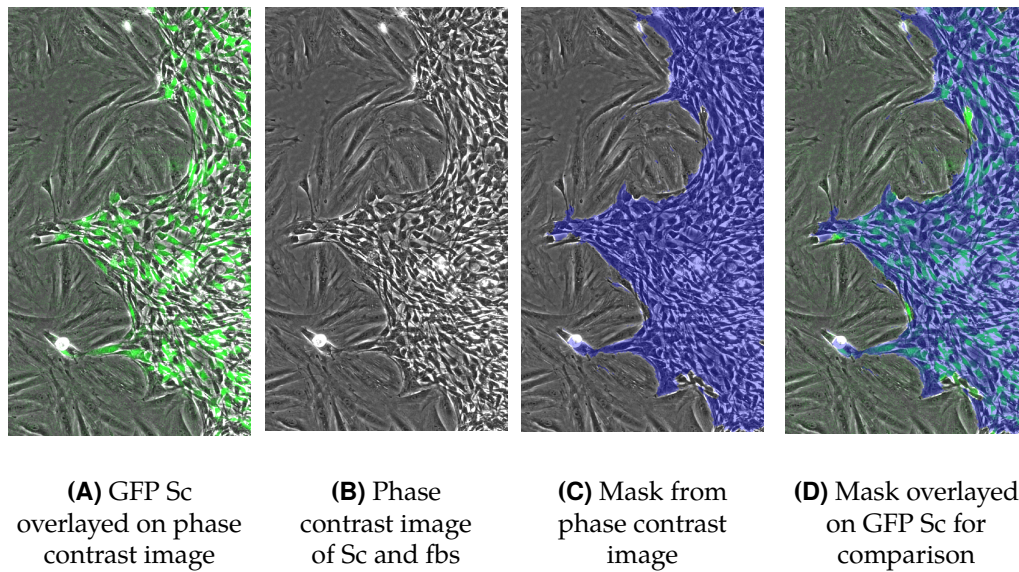


Figure 4.7: Comparing masks made from grey scale image segmentation to GFP cell labelling for identification of Schwann cells.

Using Optical Flow for Motion Extraction

Classically, measurement of displacement and thus velocity has been achieved by tracking individual cells during a time sequence. However, these tracking strategies are time-consuming, error prone and require many cells to accurately map any collective phenomenon. Moreover, manual tracking can only be carried out on sparse cell cultures, or by labelling a small percentage of the cell group. Furthermore, to understand the effects of collective cell migration it is important to go beyond the measures used for single cell migration. I use dense optic flow, a whole-field technique routinely used in computer vision to characterise and quantify the motion of objects in a video stream, often for motion-based object detection and tracking systems. Optical flow is the distribution of the apparent velocities of objects in an image. Estimating the optical flow between subsequent frames in a video, allows you to measure the velocities of objects in the video. In this case, the cells migrating relative to the stationary camera results in a flow field of motion. Dense optic flow, (as opposed to sparse optic flow) extracts a flow vector for each pixel within an image. The technique is based on the calculation of correlations between sub-windows in successive images to deduce the local displacement.

I implemented the algorithm from Deqing Su et al., ((Sun, Roth, and Black 2010; Sun, Roth, and Black 2014) in MATLAB to calculate optic flow. This method performs significantly (increased accuracy) better than classical optic flow methods on standard test sequence by using median filtering to denoise the flow after every warping step (Sun, Roth, and Black 2010; Sun, Roth, and Black 2014). The output is a vector field, where, for each pixel in the image there is a vector (D_x, D_y) which represents its velocity. This can be combined with the masks described in the previous section and visualised as a velocity map (Fig. 4.8) or as a heat map of direction (Fig. 4.9A) or of motion or speed (Fig. 4.9B).

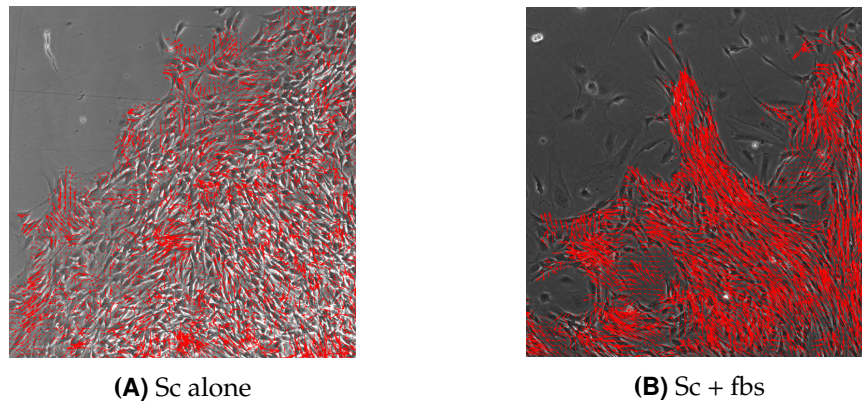


Figure 4.8: Optic flow field of Schwann cells (velocity field) over-layed on phase contrast images of (A) Schwann cells alone and (B) Schwann cells with fibroblasts. Arrows show the direction of motion and the magnitude of the arrow indicates the cell speed, larger arrows indicate a faster motion

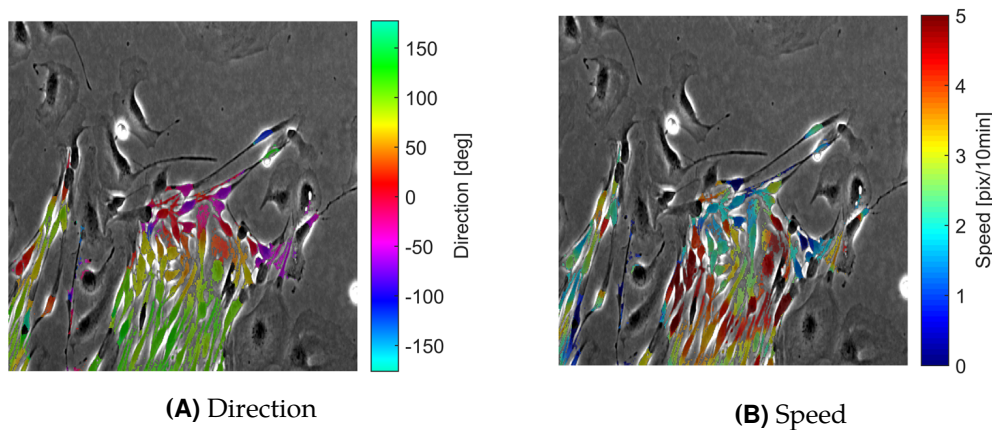


Figure 4.9: Colour-map overlaid on migrating Schwann cells (with fbs) of (A) Schwann cell direction and (B) Schwann cell speed. In (A) the colour-bar indicates the direction associated with the colour, while in (B) the colour-bar indicates the speed. Only data regarding Schwann cells is shown, the background has been masked using the methods described previously. Using optic flow combined image segmentation, the direction of motion and speed of individual cells can be seen. We can also see how the direction and speed of a given cell relate to that of its neighbours. This provides useful data for understanding how cells interact during collective migration.

Validating Optic Flow for Cell Tracking

To validate that the optic flow field accurately captures the movement of cells I com-

pared the optic flow field data to manually tracked data. Manually tracking cells is tedious and error prone and can be difficult in dense cell cultures. Therefore, I primarily tracked cells in sparse cultures in the clustering assay, or at migration front in the migration assay.

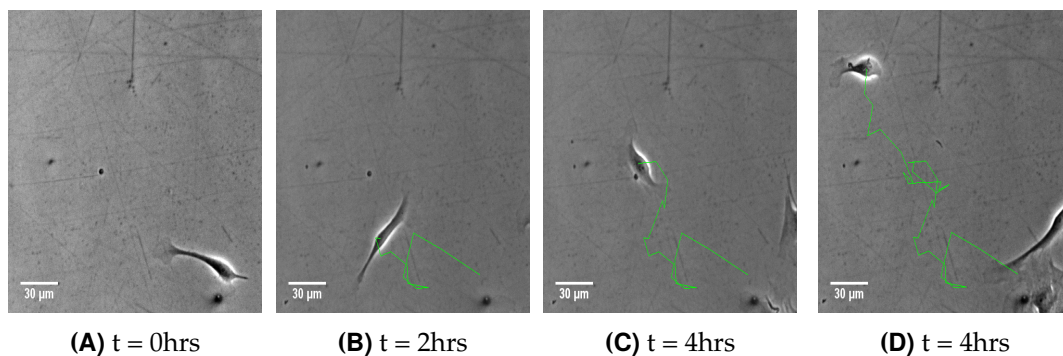


Figure 4.10: Manual tracking showing the path of a cell. At each time point ($dt = 10$ minutes), we record the xy coordinates of the cell, from which we can calculate vectors which describe the cell movement in time.

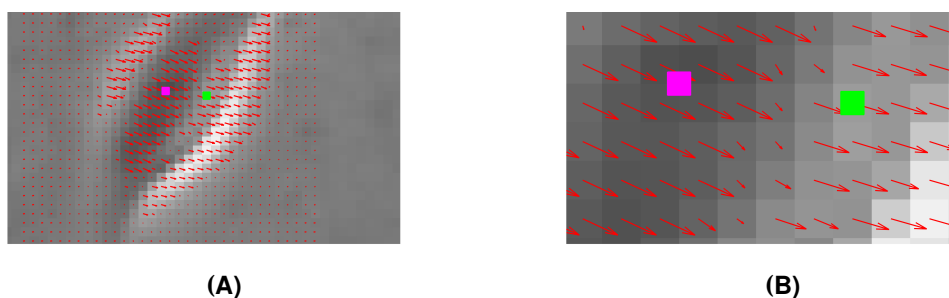


Figure 4.11: Optic flow showing movement of cell between subsequent time points. The optic flow field (for $t \rightarrow t+dt$, $dt = 10$ minutes) of the local region of interest is shown overlaid on the phase contrast image at time, t . The position of the cell determined by manual tracking is shown in pink at time t , and green at time $t + dt$. Figure b) is a zoomed in version of a).

Manual cell tracking was carried out in MATLAB using a custom written script (see Fig. 4.10). Cells were tracked by manually selecting the point in space which appears to be the centre of the cell of interest. In manual cell tracking we record a set of

Cartesian coordinates which give the spatial position of the cell for a given time, $X = ([x_1 \ y_1], [x_2 \ y_2], \dots, [x_t \ y_t])$. The direction of movement is defined as the angle between subsequent positions in space,

$$\theta = \tan^{-1}(dy/dx) \quad (4.1)$$

The optic flow of the region of interest (here the region of interest is the total area within which the cell moves for the duration of the tracking). This gives a velocity field for areas we are not interested in (i.e. the background or other cells). To select the areas of the optic flow field of only our tracked cell, I identify the area using either (a) edge detection and morphology functions in MATLAB (see Fig. 4.13) or (b) defining a square area $\sim 2 \cdot r_c^2$, (where $r_c \sim$ cell radius) centred at the point given by manual tracking (see Fig. 4.11).

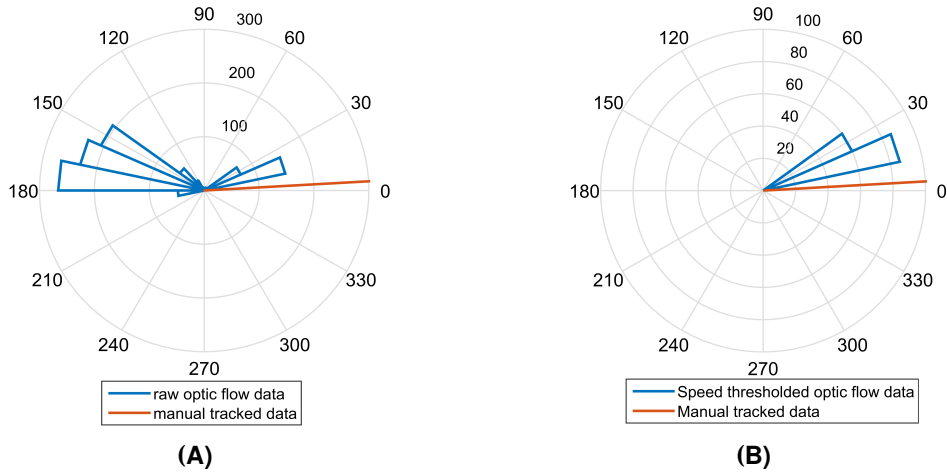


Figure 4.12: Rose plots showing the distribution of angles (blue) in the optic flow velocity field shown above in Fig. 4.11. The direction of movement of the manually tracked cell shown in orange. (A) shows raw data, and (B) shows data weighted by speed, removing noise from the background. The impact of this is the optic flow data is now significantly more similar to the manually tracked data, showing the importance of masking noise.

Manual tracking gives a set of x-y coordinates, representing the spatial position of an identifiable feature of the cell (e.g., the cell nucleus) or the estimated centre of mass over time. However, in dense optic flow the output is a velocity field where each pixel has an associated velocity vector. Furthermore, during migration (*in vitro*), not all areas of the cell are necessarily moving coherently or in the same direction; the periphery of the cell may be moving in different directions, however there is a net motion (for motile cells). The circular mean of the set of optic flow angle is compared against the single angle measured from the manually tracked data.

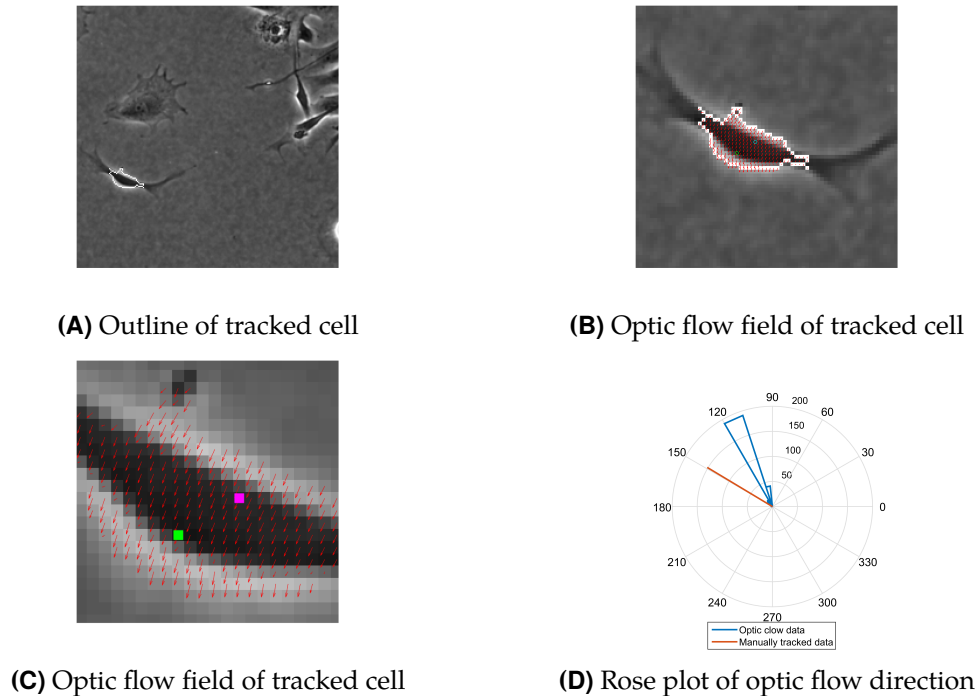


Figure 4.13: (A) The outline of the detected cell. (B) The optic flow field (for $t \rightarrow t+dt$, $dt = 10'$) of the cell overlayed on the phase contrast image at time, t . (C) The position of the cell as determined by manual tracking is shown in cyan at time t , and green at time $t + dt$. (D) Rose plots showing the distribution of angles (blue) in the optic flow velocity field shown above in (C). The direction of movement of the manually tracked cell shown in orange. Tracking only the cell body with optic flow results in a very similar direction of motion to the manual tracked motion.

The mean optic flow direction vector and manual tracking direction show a high correlation (see 4.14). This indicates that dense optical flow is a good representation of cell motion and it can be used to extract motion from live cell imaging frames. When calculating the optic flow field, the whole image is used. The velocity field from the optic flow field can be visualised as velocity map, a speed map or a direction map. In these speed and direction maps, the variable is represented in colour. For speed, cool colours represent slow speeds; warm colours represents warmer speeds.

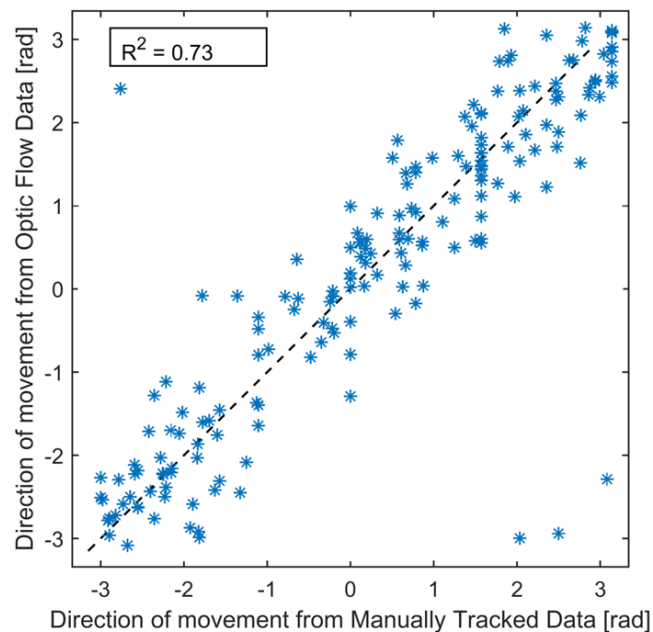


Figure 4.14: Comparing how well optic flow does compared to manual tracking in recording direction of motion of migrating cells. There is a high correlation (0.73) between the direction of motion derived from optic flow based tracking and manual tracking. This indicates that using optic flow is a good approach. In tracking motion of dense groups of cells, provided there is a good image segmentation method, optic flow is better than manual tracking as it avoids issues with human error and speeds up tracking cell motion significantly.

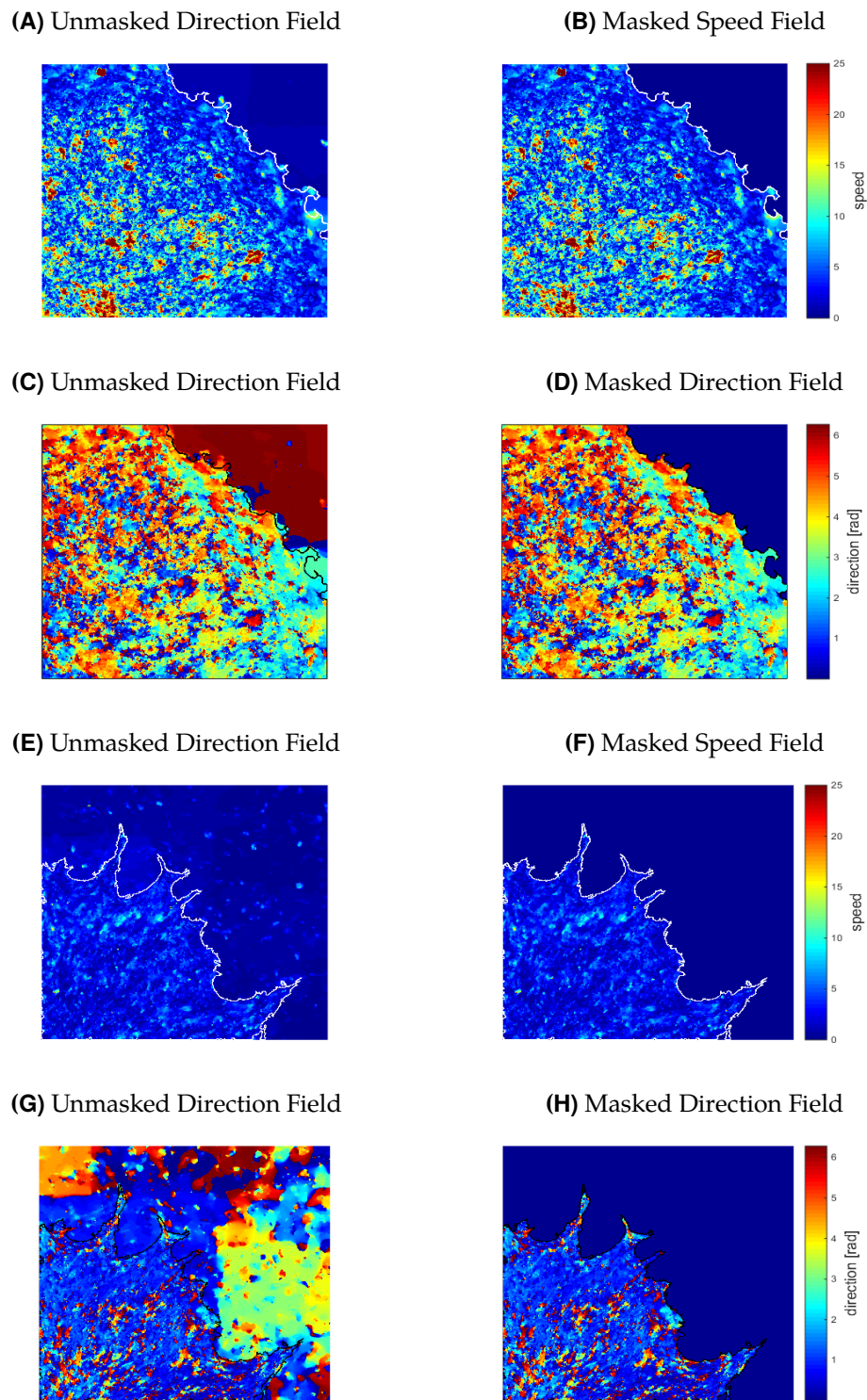


Figure 4.15: Showing how masking effects the data. The masked areas in figures (b) and (d) are set to NaN (dark blue).

Tracking Regions of Interest

In this analysis, I go beyond simple invasion/migration metrics such as time to wound closure or average speed of motion. A key challenge of this type of analysis of collective cell migration is that (b) the spatial environment is heterogeneous and (b) cells move. Due to the heterogeneous spatial environment, key or informative data can be masked, i.e. if we are interested in behaviour in a cord or at the migration front and comparing between Schwann cells migrating alone and Schwann cells migrating with fibroblasts, information from these features may be lost by being swamped by information from areas we are not interested in. To overcome this, regions of interest (ROIs) can be selected for analysis (i.e. cords or edge cells). However, this makes time series analysis challenging because as the cells move, the image content or the region of interest changes in time (see Fig. 4.16).

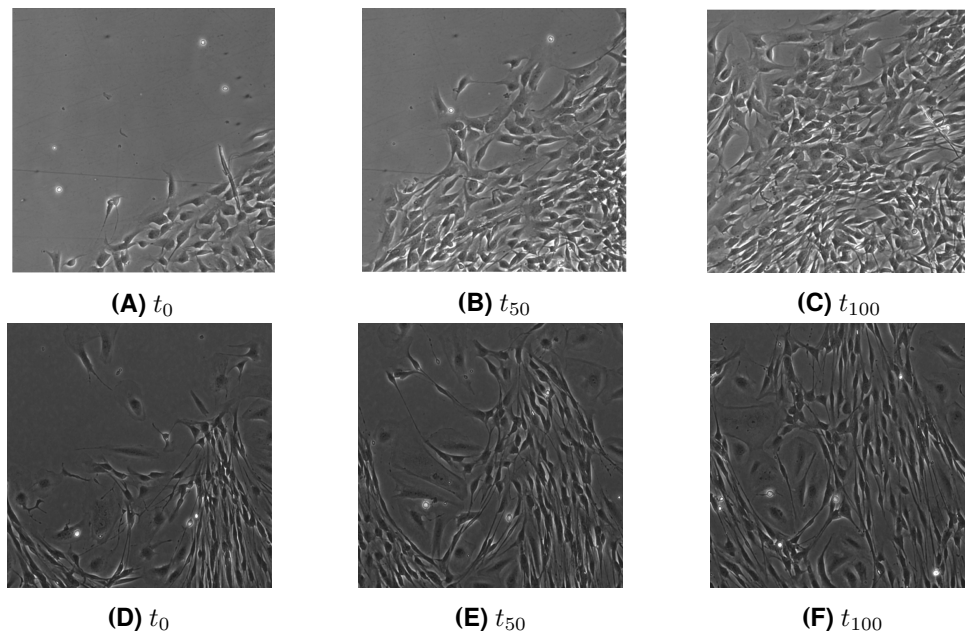


Figure 4.16: Phase contrast images of a static regions of interest (ROIs) from migration assay (top) Schwann cells alone (bottom) Schwann cells with fibroblasts. From left to right time increases, showing the significant differences in the ROI as time progresses. This makes time series analysis challenging and can make results misleading.

For time-series analysis, a method for tracking groups of cells is required. As mentioned previously, cell tracking in a dense group is challenging and time consuming. This could be overcome by using sparse fluorescent labelling, however this is not an optimal method as it is difficult to ensure the labelled cells would be in the right locations. To solve this problem I use optic flow to update the ROI. To track the position of a group of cells in frame n , the ROI position is updated according to,

$$X_{n+1} = X_n + \langle \mathbf{v}_x \cdot \mathbf{t} \rangle, \quad (4.2)$$

$$Y_{n+1} = Y_n + \langle \mathbf{v}_y \cdot \mathbf{t} \rangle \quad (4.3)$$

where X_{n+1} and Y_{n+1} are the centroid coordinates of the ROI in frame n , X_n and Y_n are the centroid coordinates of the ROI in the current frame, $\langle \mathbf{v}_x \rangle$, $\langle \mathbf{v}_y \rangle$ is the average pixel displacements in the x and y directions of the remaining optic flow field.

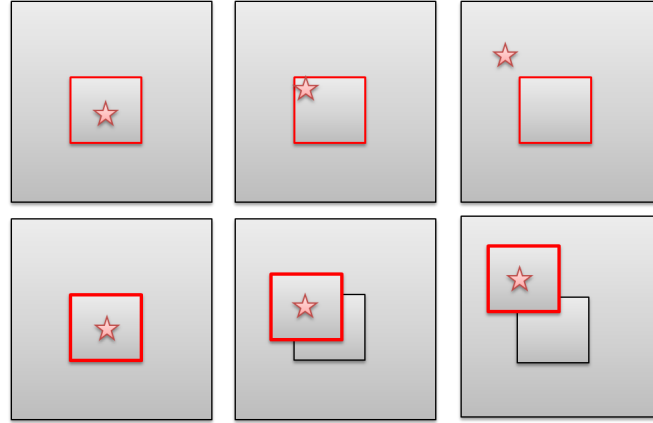


Figure 4.17: The red box indicates the ROI at each time point, with time progressing from left to right. (Top) Static ROI loses interesting features, for example, the migration front or a developing cord as the cells move out of frame. However, (bottom) a dynamic ROI moves with these features, allowing long term tracking and understanding how these features evolve.

There are uncertainties in the calculation of the global motion vectors due to noise and underestimation of optical flow in the uniform area. The object position estimated with the optical flow can be supplemented by using a template matching process. Phase correlation is used to compute the cross power spectrum between the template of the previous image and the whole current image. The cross power spectrum P between two signals in the frequency domain is defined as,

$$P = \frac{G_a \circ G_b^*}{|G_a \circ G_b^*|} \quad (4.4)$$

G_a and G_b are the original image and the template in the frequency domain, and the $*$ is the conjugate. The $\circ$ is the element-wise product. Using the cross power spectrum, the (x, y) location that produces the absolute maximum response, is where the template should be located in the background image.

Since there is no specific partition of the tracked group (i.e. contrary to tracking a solid object in a flow of water), but an arbitrarily selected area, cells can move out of the updating ROI, due to the dynamic nature of collective migration. However, this method does allow us to track the majority of a group of moving cells. This is a useful method for analysing the behaviour of a group of cells over time, and for tracking groups of cells. For example, if we want to compare how the optic flow (and measures derived from it which will be discussed later in this chapter) behave in a cord, or at the leading edge or just in a group of random cells, this method allows us to do this. Furthermore, this method provides a way to track cells to extract data such as displacement or directionality over time.

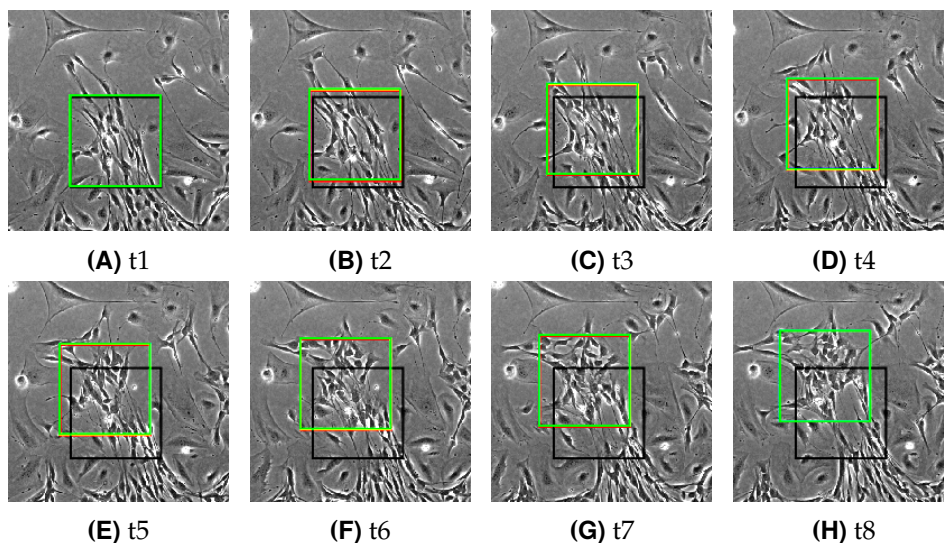


Figure 4.18: Tracking dense groups of moving cells is challenging or impossible by eye. Combining image processing methods and the underlying optic flow, local dense regions of cells can be tracked overtime. Since the cell-cell adhesions are dynamic, cells can leave and join the group. Furthermore, the tracking region is fixed, rather than dynamic. However, this method facilitates tracking groups of cells overtime, allowing for the study of how cell behaviour changes overtime, and how spatial location of cells (i.e. in a cord, or at the leading edge) impacts cell behaviour. The green box shows the dynamic updating ROI, and the black box shows the static (initial) ROI. Here a group of cells (A) breaks away from the main group of cells. These can be tracked by the green box, which follows the net motion of the cells.

Validation

To validate the tracking results, the method was applied to an area around a fluorescence labelled Schwann cell. The tracked cell was required to stay within the boundary of the ROI for all times. Schwann cells were sparsely labelled and used in the migration assay. The fluorescent images were superimposed on the phase contrast images, and the centroid position of the fluorescent cells was manually tracked, which was possible due to the sparse labelling of the Schwann cells. The region around the labelled cells was tracked using the optic flow based tracking method.

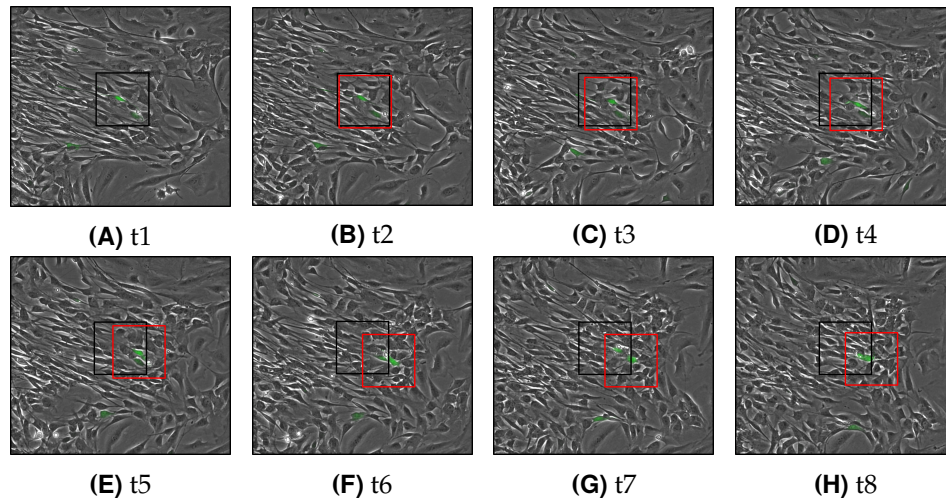


Figure 4.19: Tracking dense groups of moving cells-validation: Schwann cells were sparsely labelled using CellTracker (green cells), allowing random individual cells to be tracked over time. The performance of the cell tracking method is assessed by tracking a square region of length ten cell-radii centred on the labelled cell. If the labelled cell stays within the dynamic ROI (initial region in black, updated region in red) for the duration of tracking (minimum 1hr) the dynamic tracking is deemed to have worked well.

4.3 Measuring features of collective cell migration

The simplest analysis of the velocity field is to report the spatio-temporal dependence of the velocity. This is straightforward in the wound healing assays, (probably the most commonly used assay in CCM). It is straightforward in this setting as the group of collectively migrating cells has a very clear border. The amplitude of velocity (speed) as a function of distance to the border can be reported and in a similar way the velocity as a function of cell density. To quantify the coherence, (a key component of CCM), it is necessary to look at the direction in which cells are moving. Typically you would want to know how aligned cells are, over what length-scale and perhaps how this varies with the topology. Spatial correlations are used to measure this distance over which velocity vectors are aligned. The spatial resolution of the velocity field with respect to

cell size is very important in this case, as PIV and optic flow methods do not necessarily have the same resolution as cell size.

4.3.1 Methods

Global Entropy

I used entropy to summarise the directional field distribution. Entropy can be thought of as a measure of disorder. It has applications in many different fields, therefore it is important to be careful to define it properly. Within the field of information theory, the Shannon entropy is a measure of the uncertainty of a random variable. We can consider our vector field data as a random variable, Θ , which has a set of possible outcomes $\theta \in \theta_1, \theta_2, \dots, \theta_n$. If the probability of outcomes is known, $p(x_i)$ the Shannon entropy, $H(X)$ can be calculated as:

$$H(\theta) = -\frac{1}{\log N} \sum_{\theta_i \in X} p(\theta_i) \log_2 p(\theta_i) \quad (4.5)$$

The advantage of using entropy is that it avoids the issues which arise when calculating statistics of circular data. We can decompose our optic flow field data into two random variables; speed and direction. To calculate the Shannon entropy for a given vector field I first approximate a probability density function. To do this I construct a histogram from the data by grouping the data into a finite number of bins. The probability is then calculated as:

$$p(x_i) = \frac{C(x_i)}{\sum_{i=1}^n C(x_i)} \quad (4.6)$$

I introduce the normalizing factor ($\log N$) in order to perform comparisons between different entropies. $H(\theta)$ is 0 if all the movement direction is in the same direction.

In the opposite case, if all the movement direction is equidistributed, $H(\theta)$ takes its maximum value, Eq. 4.5. At intermediate states, $H(\theta)$ increasing as the movement direction spreads out. Data which has a more uniform distribution will have higher entropy measures, indicating higher levels of disorder. The benefit of using this method is it does not require any knowledge of the mean direction of motion. However, the drawback of this method is it does not provide any information regarding local order.

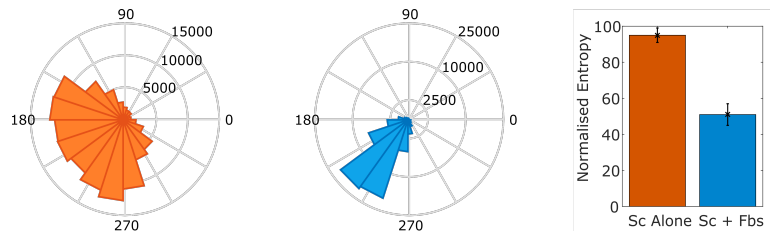


Figure 4.20: Using entropy to quantify angular data. (Left: orange circular histogram) the distribution of directions of motion of Schwann cells migrating alone. (Middle: blue circular histogram) the distribution of direction of motion of Schwann cells migrating with fibroblasts. (Right) plots of the entropy of the aforementioned angular distributions. The entropy of Sc alone (orange) is significantly higher than Sc + fbs (blue). Entropy is a concise way of summarising the differences in the angular distributions without running into issues of statistics of circular (angular) data.

Local Entropy

To study the movement of cells in local regions, I computed the entropy in the local neighbourhood surrounding each movement vector to measures of local spatial organisation. I refer to this resulting scalar field as the local entropy. The value in the entropy field for a pixel indicates the degree of vector variation in its local neighbourhood, this allows us to see areas in the field which exhibit more or less order. Given a pixel point, a local neighbourhood around the point, whose sizes are N^2 , is used to estimate the probabilities (see Fig. 4.21).

The local entropy estimates are computed from a small window, which moves over the image. A constant-size 2D local window centred at each pixel is used to compute the entropy in the pixel's neighbourhood. I discretize the velocity magnitude or direction into a certain number of bins and compute a 1D histogram for each local window accordingly. The derivation of entropy follows Equation 4.5. The entropy is high when the variability is high velocity in local regions. Intuitively, the entropy images highlight which regions in the data are important or interesting in terms of exhibiting more variation or change in their local neighbourhood compared with other regions.

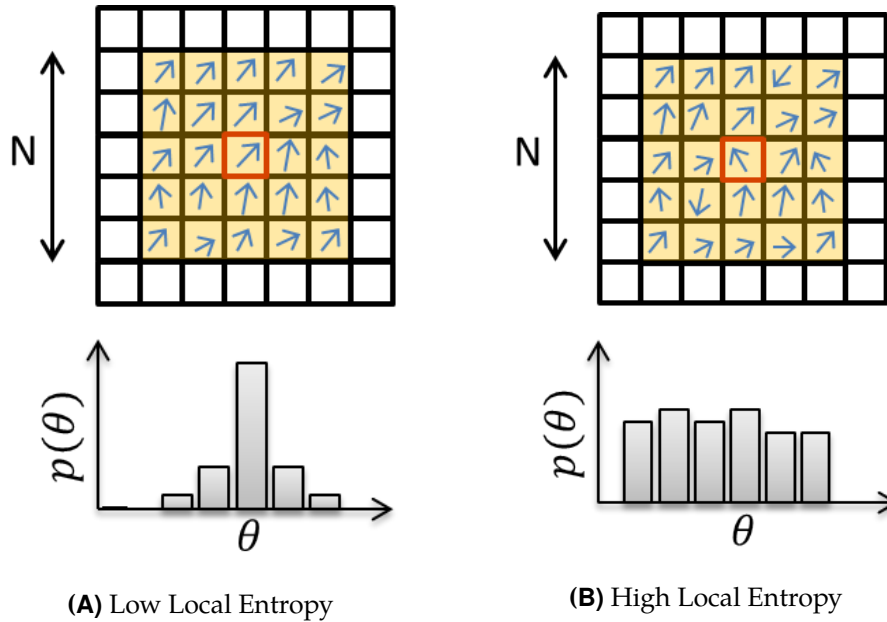


Figure 4.21: The local entropy of a given pixel is calculated by calculating the entropy of the distribution made of the histogram of direction of pixels in the $N \times N$ region around pixel.

The computation of the local entropy field requires two parameters, the number of bins and the neighbourhood size. The neighbourhood size is related to the number of pixels used to calculate the histogram for the local entropy computation, therefore the

neighbourhood size is informed by the biological length scales of cell radius.

$$H_j(\theta) = - \sum_{\theta_i \in X} p(\theta_i) \cdot \log_2(p(\theta_i)) \quad (4.7)$$

where $\theta_i = \{\mathbf{x}_i\}$. The vector field is decomposed into two scalar fields; speed and direction. For each point j in these fields I find the local entropy by creating a probability histogram of the speed or direction of the points in a local neighbourhood of size N^2 and centred at the point j . Using the local entropy field provides a method visualizing the movement of cells in a manner which is independently of actually direction of movement. Furthermore, it provides a way to identify regions of high and low order or information which I can interpret as areas of high or low alignment.

Cosine Similarity

The cosine similarity is a distance measure of similarity between two non zero vectors of an inner product space that measures the cosine of the angle between them. Given two vectors of attributes, A and B, the cosine similarity, $\cos(\theta)$, is represented using a dot product and magnitude as

$$S = \cos(\theta) = \frac{A \cdot B}{||A|| ||B||} \quad (4.8)$$

The similarity, S , is bounded by -1 and 1 . Two vectors with the same orientation have a cosine similarity of 1 , two vectors at 90° have a similarity of 0 , two vectors diametrically opposed have a similarity of -1 , and in-between values indicating intermediate similarity ($S > 0$) or dissimilarity ($S < 0$).

Mean Square Displacement

To quantify cell migration, the mean-squared displacement $\langle \mathbf{r}^2(t) \rangle$ can be calculated from position data over all experimental time. The measured cell displacement over the time interval $t_i = i\Delta t$, from position r_{t_0} to r_{t_1} is, $d_{xi} = r_{t_{x+i}} - r_{t_x}$, and the squared displacement is, $\mathbf{r}_{xi}^2 = d_{xi} \cdot d_{xi}$. When multiple sets of migration trajectories are available, the time averaged squared displacement (TASD) can be calculated to reduce noise. The TASD of the i^{th} migration path is given by

$$\langle \mathbf{r}^2(\tau) \rangle = \langle [\mathbf{r}(t + \tau) - \mathbf{r}(t)]^2 \rangle \quad (4.9)$$

where, $\mathbf{r}(t)$ is the position of a cell at a given moment in time, t and $\mathbf{r}(t + \tau) - \mathbf{r}(t)$ is the distance travelled by a cell in time τ .

The behaviour of collectively migrating cells (Marel 2014) has been shown to exhibit significant deviations from the linear time dependence of the mean squared displacement $\langle \mathbf{r}^2(t) \rangle \sim \tau$ which is characteristic of Brownian motion. Instead, anomalous diffusion (Jean-Philippe Bouchaud 1990; Metzler and Klafter 2000; Metzler and Klafter 2004) better describes the observed trajectories. Anomalous diffusion is of the form,

$$\langle \mathbf{r}^2(\tau) \rangle \sim D\tau^\alpha \quad (4.10)$$

where D is the diffusion coefficient, τ is the elapsed time, and the exponent α characterises the type of motion. In a typical linear diffusion process, $\alpha = 1$, $\alpha = 0$ indicated no motion, and $\alpha = 2$ indicates ballistic motion. Other values represent sub-diffusive motion $0 < \alpha < 1$, or super-diffusive motion $1 < \alpha < 2$. Super-diffusion can be the result of active cellular transport processes.

There are two main methods used to quantify the average diffusive behaviour of cells. The conventional approach is the ensemble averaged (EA) MSD. The EA MSD is defined from the trajectories $r_i(\tau)$ of the moving cells (each cell is denoted with the index i) in terms of the distance from their initial position $r_i(0)$,

$$\langle \mathbf{r}^2(\tau) \rangle = \frac{1}{N} \sum_i^N (\mathbf{r}(t + \tau) - \mathbf{r}(t))^2 \quad (4.11)$$

Where N is the total number of measured cells. Alternatively, the diffusion of an individual cell can be quantified from the single trajectory $r(t)$ using the time averaged (TA) MSD (Andreanov and Grebenkov 2012; Barkai, Garini, and Metzler 2012) as

$$\chi_{\tau,T}^2 = \frac{1}{T - \tau} \int_0^{T-\tau} (\mathbf{r}(t + \tau) - \mathbf{r}(t))^2 dt \quad (4.12)$$

where τ is the lag time and T is the total time. The TA MSD is constructed from the particle displacement during the time lag τ accumulated over the entire trajectory.

4.3.2 Results

Measuring Migration Speed

To look at the speed of motion of cells the speed component of the optic flow field can be visualised. Warm colours represent cells moving in faster directions, while cooler colours represent slow speeds (see Fig. 4.22 for representative images).

The speed profile (histogram of speed data) for each optic flow field overtime can be described using a gamma distribution (see Fig. 4.23B). Parameters for the gamma

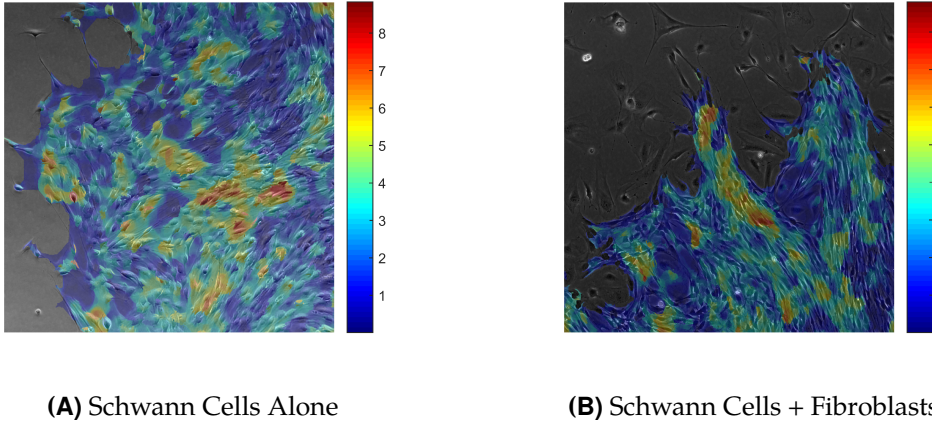


Figure 4.22: Phase contrast images of the migration assay with speed (derived from the optic flow field) overlaid. The colour-bar shows speed in $\mu\text{m/hr}$ (A) Schwann cells alone (B) Schwann cells with fibroblasts.

distribution from the (raw) experiment speed data were calculated using the maximum likelihood estimates (MLEs). To check that the gamma distribution is a good fit for the data I look at the adjusted R^2 value. The gamma distribution can be parameterised in terms of a shape parameter $\alpha = k$ and an inverse scale parameter $\beta = 1/\theta$, called a rate parameter. A random variable X that is gamma-distributed with shape α and rate β is written as,

$$f(x) = \frac{\beta^\alpha x^{\alpha-1}}{\Gamma(\alpha)} \exp^{-x\beta} \quad (4.13)$$

I found that Schwann cells alone and Schwann cells with fibroblasts both have very broad speed distributions (Fig. 4.23). Interestingly, Schwann cells alone exhibit a broader speed distribution, indicating a faster speed than Schwann cell with fibroblasts, even though the average speed of Schwann cells in a monoculture in a sparse setting is significantly lower than Schwann cells in co-culture with fibroblasts. This is indicative of collective motion, and can also be in part due to the presence of fibroblasts, which

spatial restrict cell motion of Schwann cells.

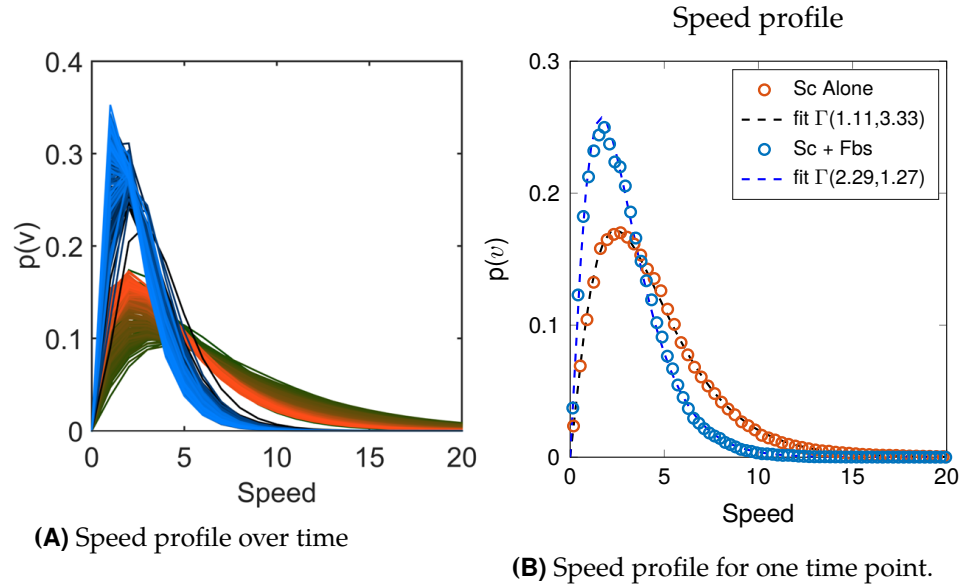


Figure 4.23: Speed profile of Schwann cells migrating alone (orange) and with fibroblasts (blue). (A) Line color hue (dark to light) represents time, showing that when Schwann cells are in the migration assay alone, the speed profile decreases in broadness over time, while when Schwann cells migrated with fibroblasts the speed profile is maintained. (B) A gamma distribution fit to the speed profile.

Relating Speed and Local Entropy

Transforming the optical flow field to the local entropy field allowed me to look at how speed related to local correlation (see Fig. 4.25). There is a negative correlation between speed and local entropy (high local entropy indicates high disorder). The magnitude of the correlation coefficient between speed and local entropy for Schwann cells alone is smaller than for Schwann cells with fibroblasts (see Fig. 4.24C). By looking at examples of the joint probability distributions of speed and local entropy, (for examples see Fig. 4.24A and Fig. 4.24B) and the distribution of correlation coefficients, it is clear that high disorder can occur with any speed. Connected areas of low local entropy and associated higher speeds were typically identified in cord like protrusions in the migration assays where fibroblasts were present. This indicated that the cord like migration was facilitated by the presence of fibroblasts which result in local cohesion between cells. It also suggests that although the mean speed of migration when Schwann cells are alone is greater than when with fibroblasts, areas in which cords are forming have a higher speed than those not in cords.

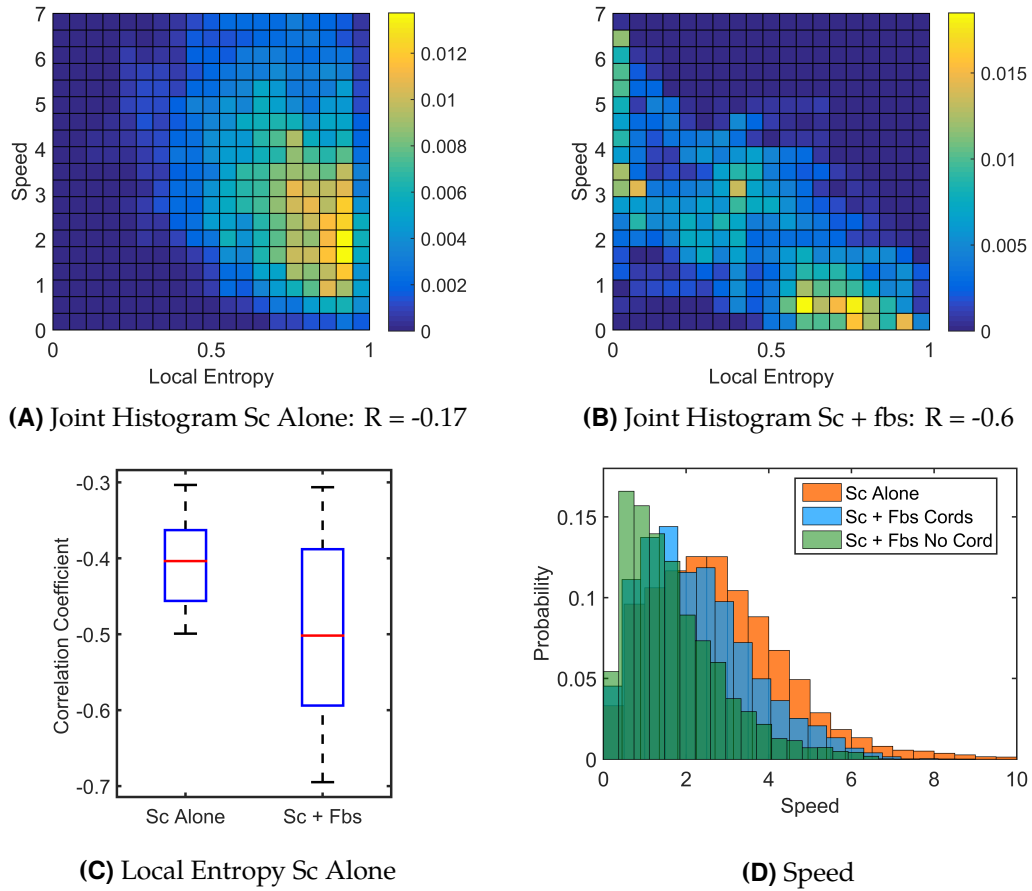


Figure 4.24: (A,B) Joint histogram of speed and local entropy for Sc alone and Sc + fbs respectively, shows that lower local entropies (higher local correlation) are associated with higher speeds. (C) Correlation coefficient of speed and local entropy. (D) Histogram of speed for Schwann cells alone (orange), for Schwann cells + fbs in areas where cords are forming (blue) in areas where cords are not forming (green).

Measuring Migration Direction

Fibroblasts increase global order in migrating Schwann cells

To look at the direction of motion of cells the directional component of the optic flow field can be visualised. Similar colours represent cells moving in similar directions, while contrasting colours represent different directions. As can be seen in Figure 4.26, when Schwann cells are with fibroblasts the direction of motion is highly similar

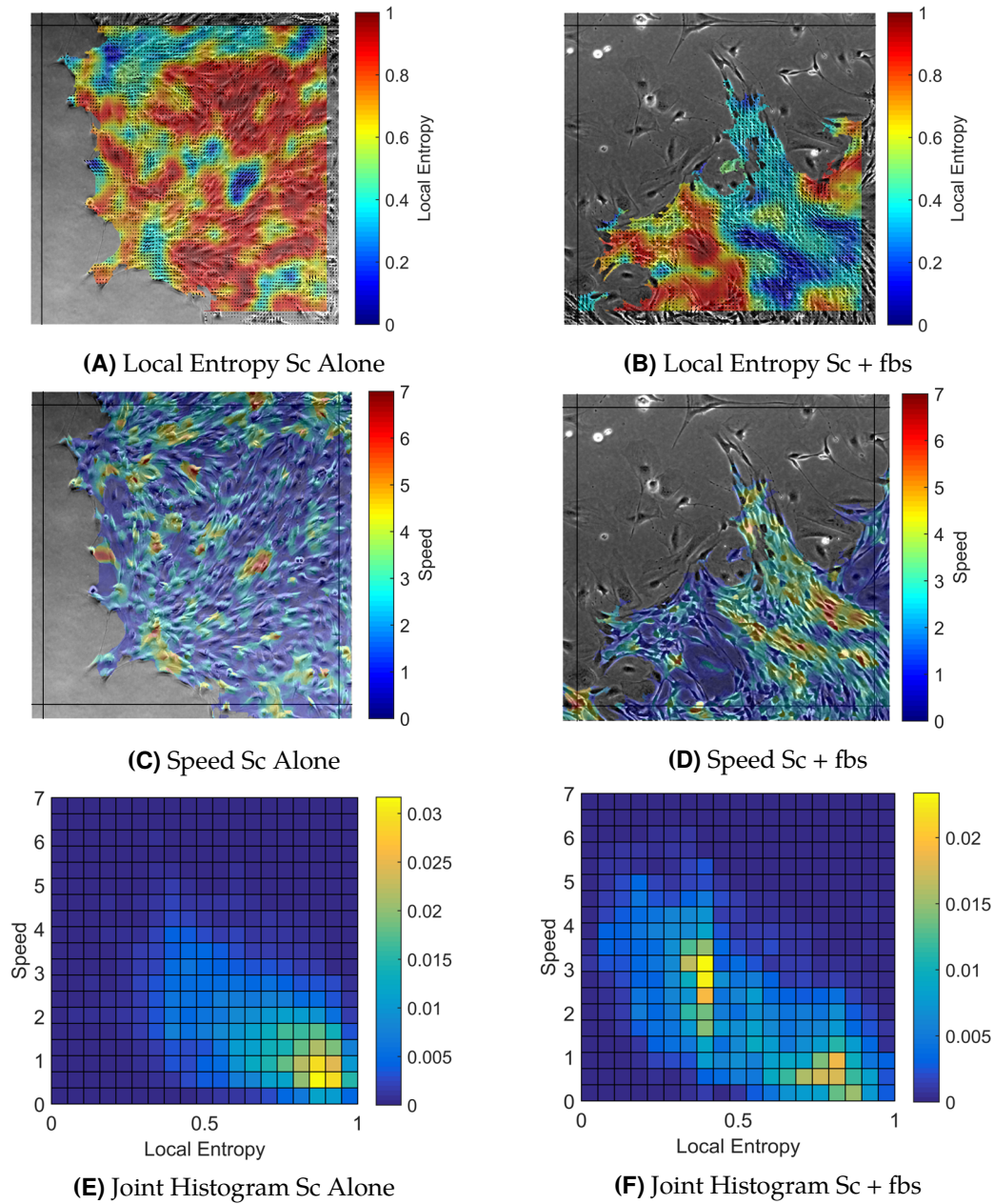


Figure 4.25: Phase contrast images of the migration assay with speed (C,D) and local entropy (A,B) overlaid. Joint histogram of speed and local entropy shows that lower local entropies (higher local correlation) are associated with higher speeds.

between local cells, while when Schwann cells migrate without fibroblasts present, the direction of motion is more random between cells. The directions can be summarised

in a circular histogram, which show the aggregate directionality distributions for either one time frame or for all time. The distribution of directions for Schwann cells alone 4.26 top right (orange), is much more uniform than the distribution of directions for Schwann cells with fibroblasts 4.26 bottom right (blue).

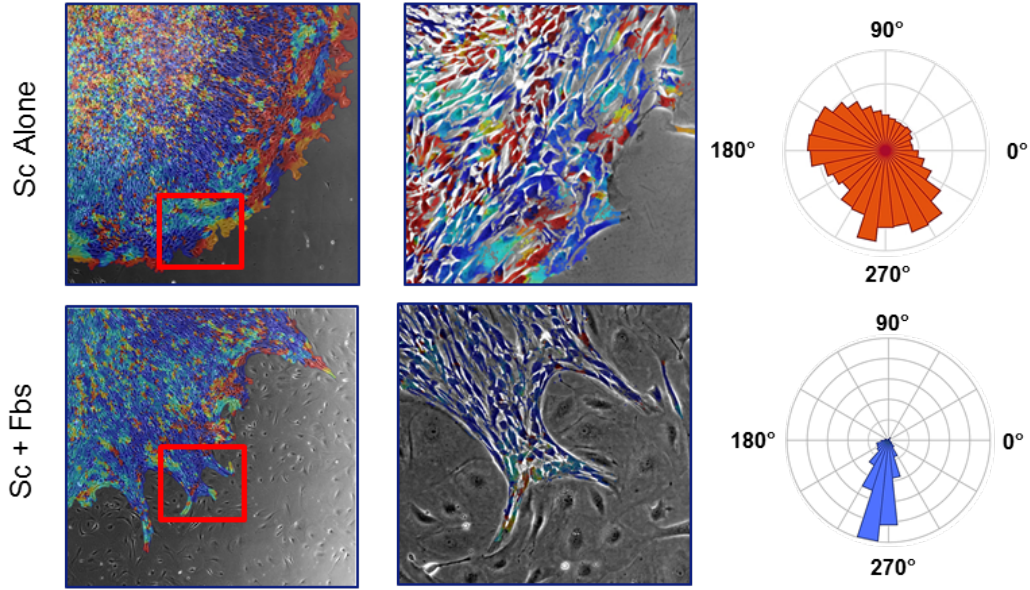


Figure 4.26: (Left and middle) Optic flow directional component overlaid on phase contrast images of snapshot of the migration assay for (top) Schwann cells alone and (bottom) Schwann cells with fibroblasts. The direction field for Schwann cells alone is very colourful, indicating many different directions of motion, while the direction field for Schwann cells with fibroblasts is predominantly blue, indicating a the majority of the cells are moving in the same direction. (Right) Circular histograms of aggregate cell directions for (top) Sc alone and (bottom) Sc + fbs (data from images shown in middle images).

Using entropy of the directional component of the velocity field is a means to quantifying global order. Schwann cells alone have a higher entropy (degree of disorder) than Schwann cells with fibroblasts. Furthermore, as time progresses, the directional entropy of Schwann cells alone increases, whereas when Schwann cells are with fibroblasts, their directional entropy stays the same or can even decrease (see Fig. 4.27).

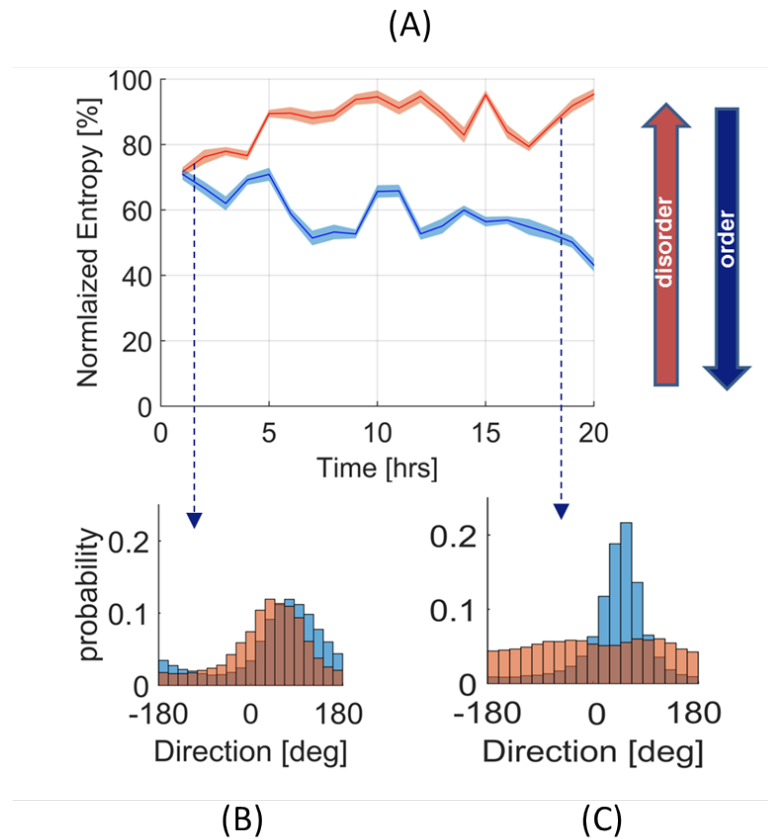


Figure 4.27: Entropy as a readout of global order. (A) Directional entropy of migrating Schwann cells cultured alone (orange) and co-cultured with fibroblasts (blue). Schwann cells are plated alone or with fibroblasts in the migration assay and allowed to migrate out. As time progresses the entropy increases in Schwann cells which are cultured alone (indicating a decreases in migration order) but decreases in Schwann cells co-cultured with fibroblasts (indicating an increase in order). Histograms of direction of motion initially (B) and after 18hrs(C). Initially the distribution of direction is similar for Schwann cells alone and Schwann cells with fibroblasts (weakly focussed at 70°), however overtime, for Schwann cells with fibroblasts, this distribution becomes concentrated about the initial direction of motion whereas the direction distribution for Schwann cells alone flattens out.

Fibroblasts increase local order in migrating Schwann cells

Using local entropy, I looked the effect of fibroblasts on the local order or local alignment of migrating Schwann cells. Local entropy provides a direction invariant mapping of direction, as well as a readout of local coherence at different length scales. As described earlier, the local entropy is a function of the neighbourhood size and the number of bins used in the histogram. By keeping the number of bins constant and varying the neighbourhood size (N), the local coherence at different length scales can be explored. For example, using $N = 2r_c$, where r_c is a cell radius gives information of the coherence within an area of roughly 2 cells. As can be seen in Fig. 4.28, Schwann cells migrating with fibroblasts exhibit large continuous areas of coherence, while when alone there is little coherence between neighbouring cells. In Fig. 4.28B, there is a clear localised area of high local entropy (low order) at a point on the leading edge where a cord is forming.

The local coherence is summarised in a histogram 4.29A, which shows a clear shift towards zero when Schwann cells are with fibroblasts compared to when they migrate alone. The median of the data can be used as a readout for the local coherence at a given neighbourhood size, and by increase N I see that the local coherence when Schwann cells are alone increase to 1 much more quickly than when Schwann cells are with fibroblasts (see Fig. 4.29B. This shows that even though interactions with fibroblasts (via CIL) can disrupt local coherence, they also increase the length scale over which local coherence is maintained.

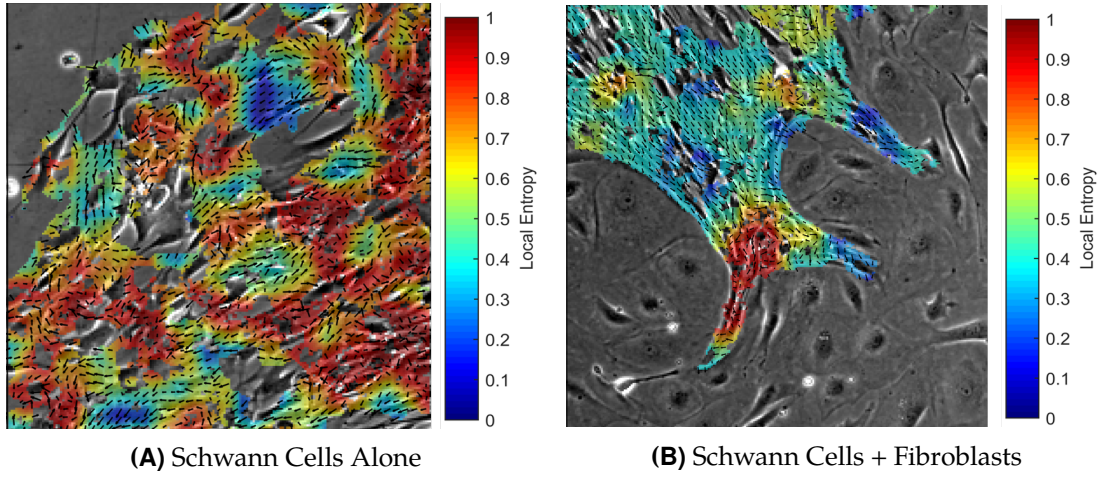


Figure 4.28: Phase contrast images of the migration assay with local entropy ($N = 2$ cell radii) overlaid. Higher values of local entropy (warmer colours) indicate disorder and low values (cooler colours) indicate order. (A) Schwann cells alone; large regions of red indicate predominately local disorder at this length scale. (B) Schwann cells with fibroblasts, large regions of blue/green indicate predominately local order at this length scale, however at the leading edge (where interaction with fibroblasts are common), transient disorder occurs (red region).

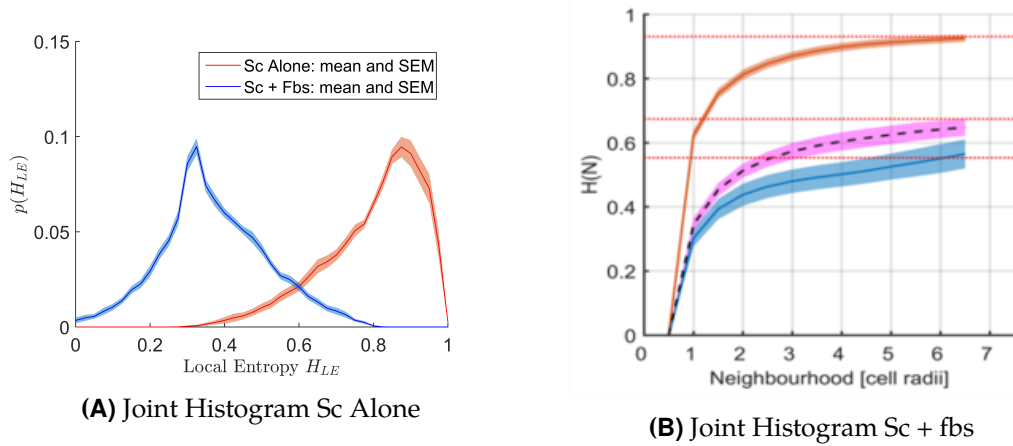


Figure 4.29: (A) Histograms of local entropy for Schwann cells alone (orange) and Schwann cells with fibroblasts (blue) are used to summarise the local entropy field. (B) The median value of the distribution as a function of neighbourhood size for Schwann cells alone (orange), Schwann cells with fibroblasts in cords (blue) and Schwann cell with fibroblasts not in cords (pink).

Persistence and Directional Motion

To study persistent motion I looked at how the directional optic flow field evolved in time. Randomly moving cells can exhibit persistent motion on a short time frame, so I compare optic flow derived from images at $OF(t, t+1)$ and $OF(t+2, t+3)$. Schwann cells with fibroblasts maintain the directional motion clearly in large areas compared to Schwann cells alone (see figure 4.31), however on occasion I observe a transient loss of persistence in areas at the leading edge where Schwann cells have come into contact with fibroblasts and some dynamic re-arrangement of the local group of cells occurs (see figure 4.31D and 4.31D circled in red.).

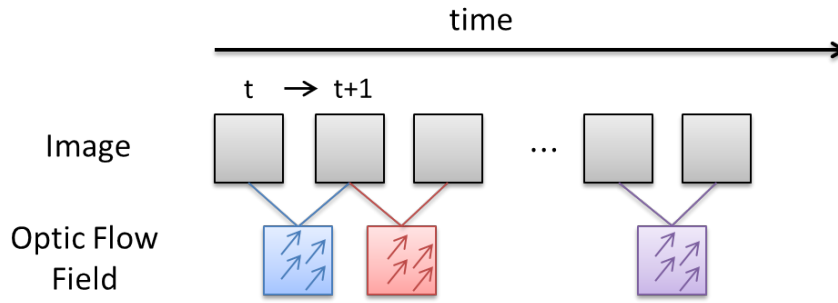


Figure 4.30: Schematic showing how optic flow is related to image sequence.

To quantify the similarity in flow fields I use the cosine similarity, $S = \cos(\theta)$, where θ is the difference between two flow fields (see Fig. 4.32). The similarity is bound between $[-1, 1]$, -1 meaning exactly opposite, and 1 meaning exactly the same, with 0 indicating orthogonality, and in-between values indicating intermediate values of similarity ($S > 0$) or dissimilarity ($S < 0$). To summarise the similarity over time, I look at the percentage of area under the histogram greater of equal to $S = 0.5$. Since the cells are migrating, the region of interest (ROI) at $t = 1$, is very different at $t = 100$, see Figure 4.16), and means this means comparing $S(t, t+dt)$, and $S(t+DT, t+Dt+dt)$ can be misleading. Tracking a ROI shows that the Similarity of a region is relatively

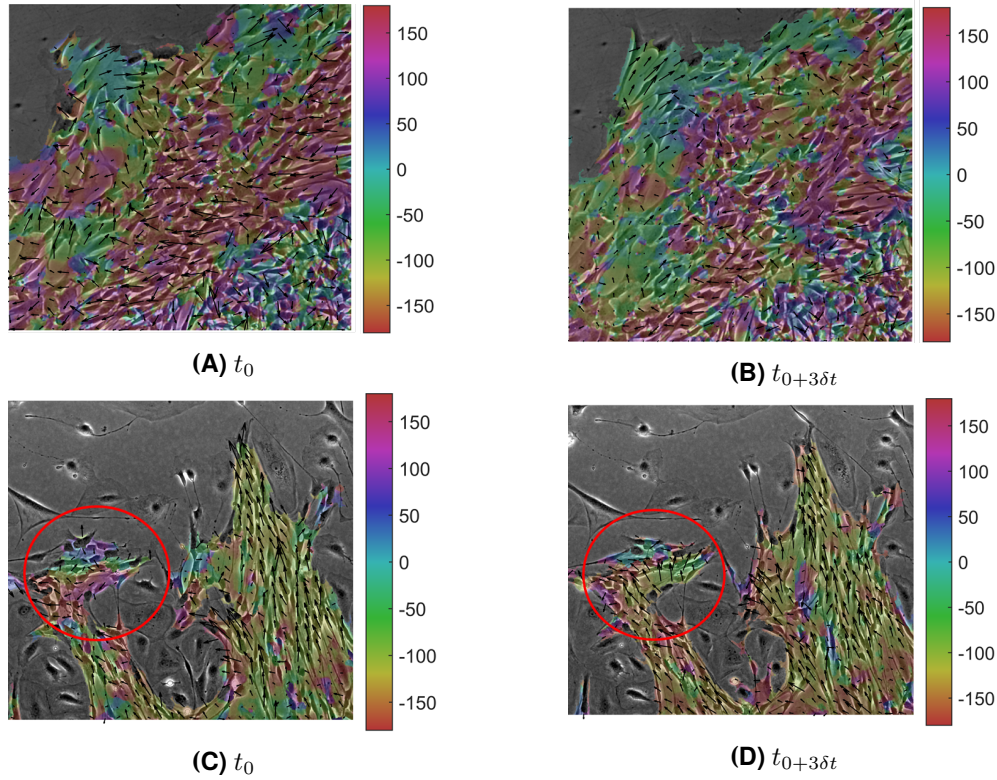


Figure 4.31: Phase contrast images of the migration assay with direction (from optic flow field) overlaid (top) (A) Schwann cells alone at time t , (B) Schwann cells alone at time $t + 3*dt$, (bottom) (C) Schwann cells with fibroblasts at time t , (D) Schwann cells with fibroblasts at time $t + 3*dt$. Significant changes in direction of motion are observed when Schwann cells are alone, whereas when Schwann cells are with fibroblasts the majority of the cells maintain the motion. Circled in red is a region where there is significant deviation from this pattern. This is due to Schwann cells interaction with fibroblasts and due to CIL undergoing repulsion and a reorganisation within the cohesive cell group. Evidence for this is that the cell group changes direction and reforms the cord around the group of fibroblasts.

constant (small decrease over time) for both Schwann cells alone and Schwann cells with fibroblasts, and the similarity is significantly higher when fibroblasts are present (see Fig. 4.33).

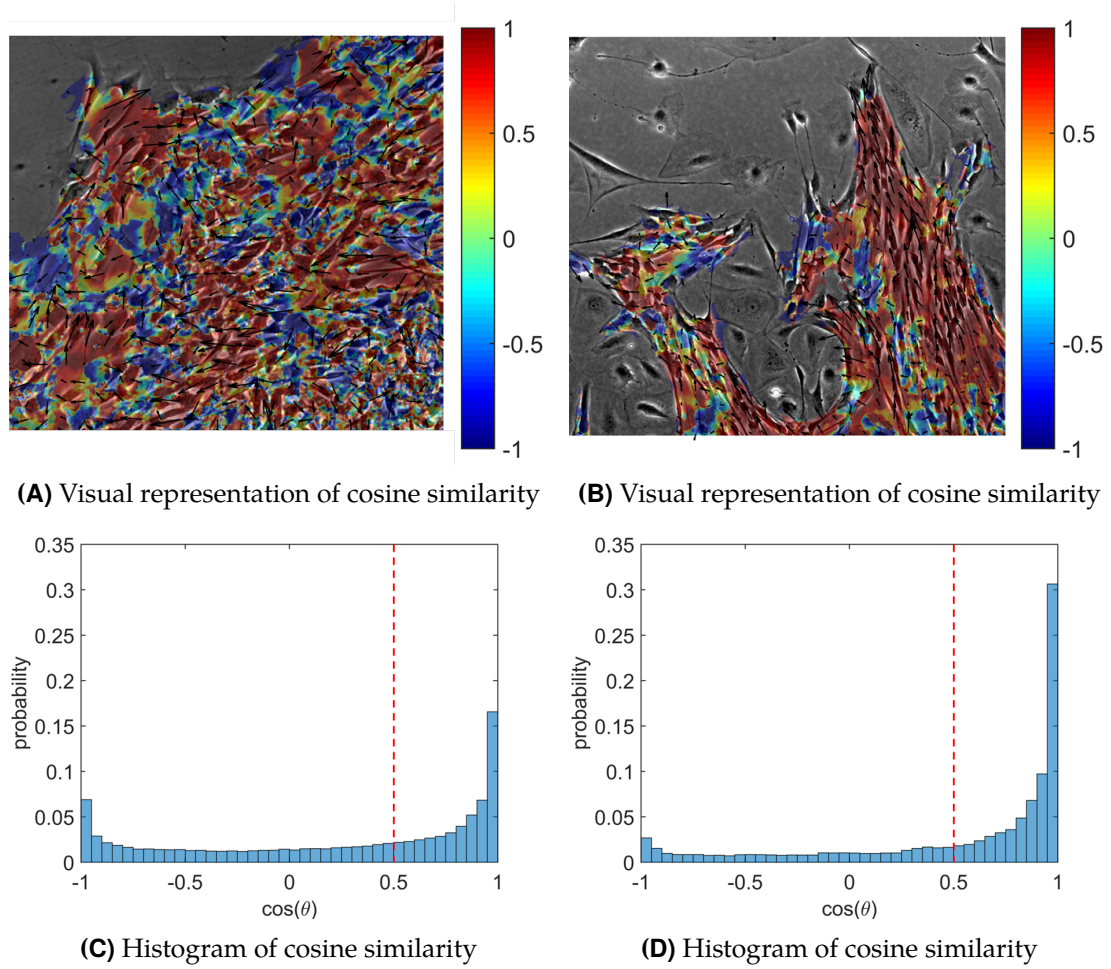


Figure 4.32: (Top) Phase contrast images of the migration assay with cosine similarity overlaid for (A) Schwann cells alone and (B) Schwann cells with fibroblasts. (Bottom) of cosine similarity (C) Schwann cells alone and (D) Schwann cells with fibroblasts. The similarity is the cosine of the difference between the velocity fields, $S = (\cos\theta)$, values greater than zero indicate similarity with $S = 1$ indicating no change, and values less than zero indicate dissimilarity with $S = 0$ indicating 180 degrees change. Although when Schwann cells are alone there are regions of high similarity (red), the regions are disjointed, whereas when Schwann cells are with fibroblasts there are large regions of continuous high similarity. This is reflected in the histograms (bottom), where the distribution is shifted towards 1 for Schwann cells with fibroblasts compared to Schwann cells alone.

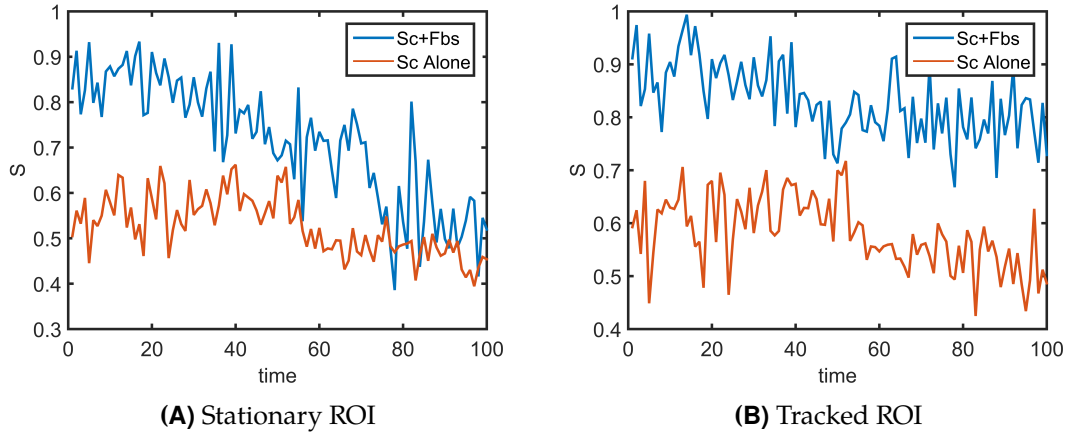


Figure 4.33: Cosine similarity - a similarity measure for measuring persistence in motion. Over time for (A) stationary ROI and (B) updating the region of interest (ROI). When tracking the ROI, the similarity, S is higher when Schwann cells are with fibroblasts, indicating a higher persistence in motion. However, this result (for long periods of time, i.e. sufficient time for the cells to move out of the ROI) is even more clear when tracking groups of cells by updating the ROI as cells migrate.

Directionality and MSD

To characterise how cells migrate overtime, I studied the directional persistence of groups of cells. To do this, I approximated the mean square displacement $\langle r^2(t) \rangle$, of a group of cells at the leading edge by using the updating ROI method described earlier. The relationship between MSD and time t is written as; $\langle r^2(t) \rangle \sim t^\alpha$. For $\alpha < 1$, $\alpha = 1$, and $1 < \alpha < 2$, the motion is said to be sub-diffusive, normal diffusive and super-diffusive, respectively. For $\alpha = 2$, the motion is ballistic, i.e., persistent and directed one dimensional motion, while diffusive behaviour describes a persistent random walk. When Schwann cells migrated alone, for a group of cells at the leading edge, $\langle r^2(t) \rangle$ was diffusive or near sub-diffusive, i.e., $1 \leq \alpha \leq 1.3$, while when Schwann cells migrated with fibroblasts $\langle r^2(t) \rangle$ was mostly near ballistic, i.e., $1.7 \leq \alpha \leq 2$ (Fig. 4.34A). This confirms that, in spite of contact inhibition of locomotion (CIL) between Schwann cells and fibroblasts, the fibroblasts increase the persistent displacement of groups of cells. Another measure of persistence is directionality, which is defined as the displacement

divided by the total length of the cell trajectory. When a cell is migrating randomly, directionality decreases and when a cell is migrating in a directional way, directionality increases.

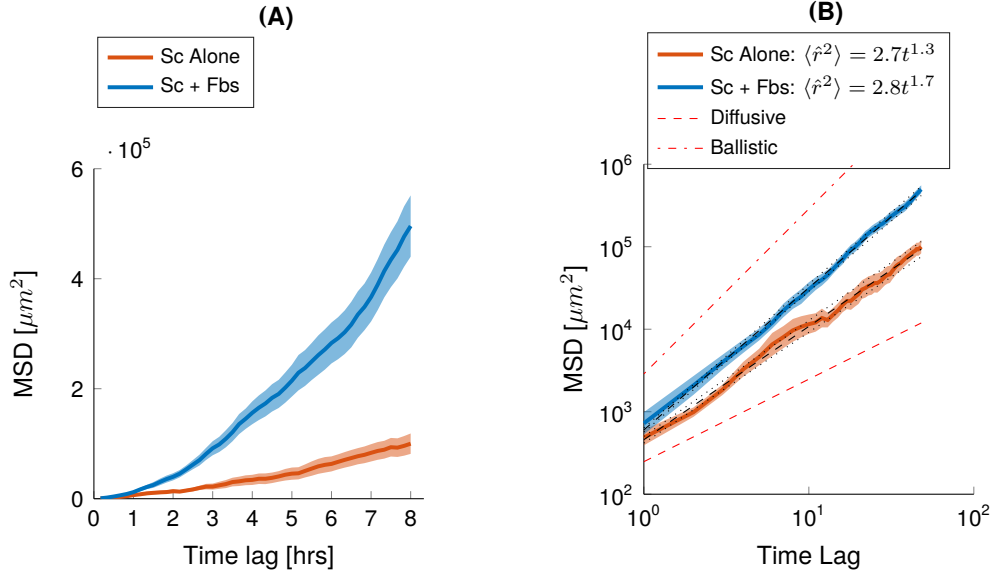


Figure 4.34: The mean squared displacement $\pm$ SEM shown in (A) linear and (B) log scale plots. (A) Shows the non-linear relationship between space explored and time, implying the diffusion is anomalous. The MSD(t) of Sc in the migration assay with and without fbs is shown in blue and orange respectively. (B) The MSD(t) of individual cells in the migrating sheet in both cases is super-diffusive. The exponent parameter for Sc + fbs is significantly higher than Sc Alone. This shows that in spite of CIL and repulsion from fbs, the Sc + fbs migration is more directional than Sc alone. Reference lines for diffusive ($\alpha = 1$) and ballistic motion ($\alpha = 2$) are shown.

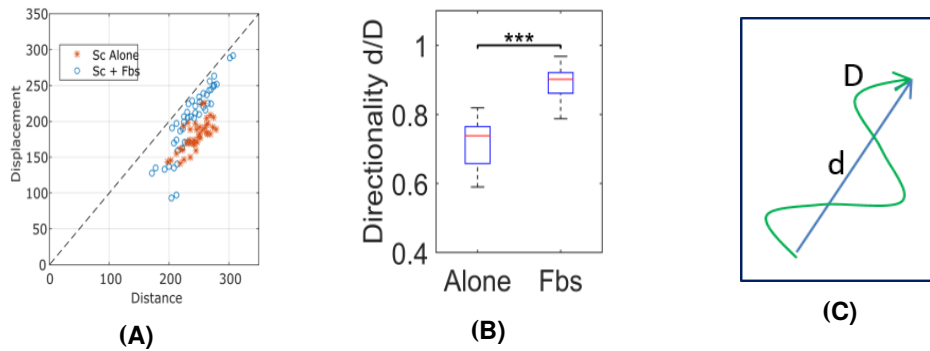


Figure 4.35: Directionality can be used as a measure of persistence, it is defined as the displacement divided by the total length of the cell trajectory. When a cell is migrating randomly, directionality decreases, while when a cell is migrating in a directional way, directionality increases - as shown in the cartoon in (C), where the displacement is denoted as d , and distance travelled as D . (A) Displacement as a function of distance travelled is plotted for both Schwann cells alone and Schwann cells with fibroblasts, (B) box-plots of distance/directionality show Schwann cells migrating with fibroblasts are significantly more directional than those migrating alone, $n = 50$.

Cell-Cell Interactions

Cell-cell interactions are very hard to track and understand in dense collective migration. The cell-cell interactions I was interested in were contact inhibition of locomotion between Schwann cells and Schwann cells, and contact inhibition of locomotion between Schwann cells and fibroblasts, see Fig. 4.36 for examples.

The optic flow field can be used to clarify cell behaviour following interactions with other cells. Typically, this is done by manually tracking cells, as shown in Fig. 4.36. This shows us incidents of homotypic contact inhibition of locomotion (Sc-Sc), higher adhesion between Schwann which are migrating and have come into contact with fibroblasts, which acts to decrease the effect of CIL between cells, and heterotypic CIL between Schwann cells and fibroblasts, causing Schwann cells to change direction. These behaviours can be looked at using the optic flow, as shown in Fig. 4.37. The directional field of motion can show not only the resultant motion of tracked cells, but

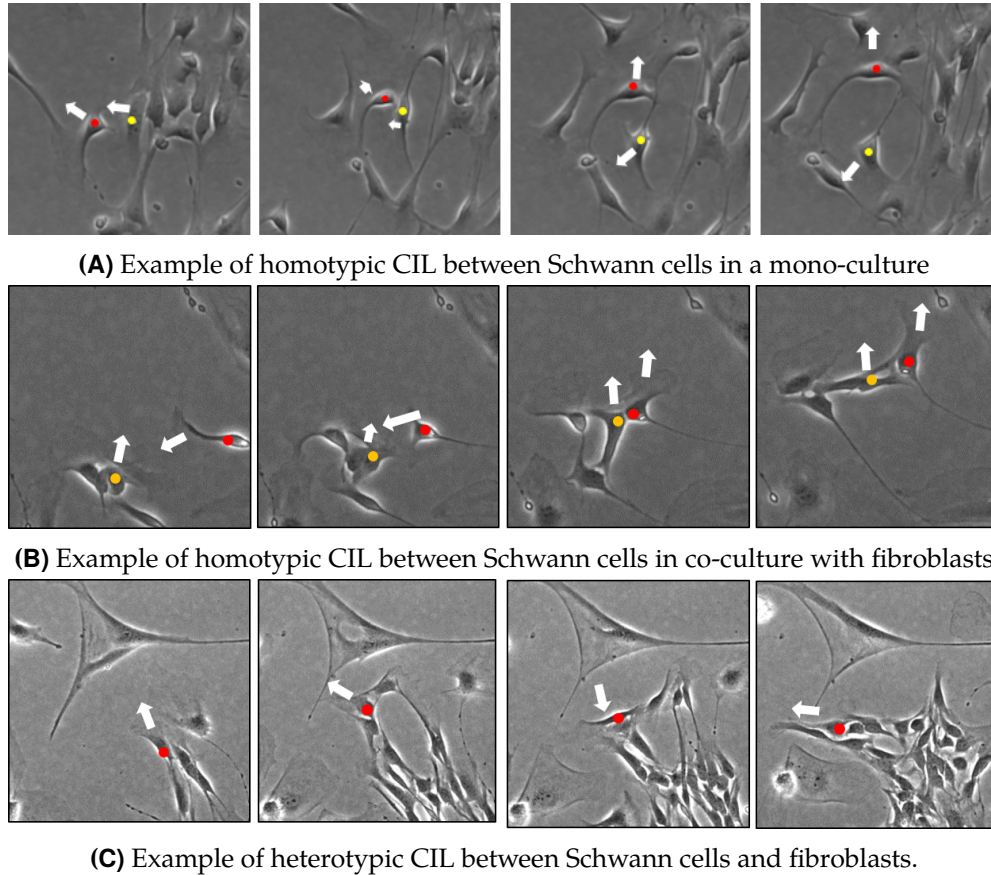


Figure 4.36: Examples of different cell-cell interactions in phase contrast images. Snapshots of migrating cells are shown at time intervals of 40 minutes, white arrows denote direction of motion. (A) Example of homotypic CIL between Schwann cells in a mono-culture. Two Schwann cells (yellow and red dot) come into contact and subsequently move off in opposite directions after coming into contact. This is due to a contact inhibition of locomotion interaction occurring between the two cells. It results in the cells separating out, and not forming long lasting groups. In the 2D culture assay it also accounts for why the spatial organisation of cells is randomly or uniformly (depending on density) distributed in space. (B) Example of homotypic CIL between Schwann cells in co-culture with fibroblasts. Two Schwann cells (yellow and red dot) come into contact. These cells are migrating in the presence of fibroblasts and have previously come into contact with them. After coming into contact with each other, although the cells do not appear to immediately adopt the same direction of motion, they also do not separate and connection is maintained between the cells. (C) Example of heterotypic CIL between Schwann cells and fibroblasts. Interactions between Schwann cells and fibroblasts typically lead to Sc-fbs repulsion via contact inhibition of locomotion. A manually tracked Schwann cells (red dot), reverses and then moves to the left after interacting with a fibroblast. Some mechanism, either CIL or volume exclusion between the following Schwann cells forces the labelled cell to move to the left where there i.e. free space.

those around them. In this example, the leader cells of a forming cord undergo CIL with a fibroblasts, resulting in the cells changing direction and moving off around the fibroblast. To better understand the way in which groups of cell migrate I tracked groups of cells (~ 30) using my tracking ROI method. For each ROI I calculated the entropy and used this as the readout of order within the cells. I noticed that the variance in entropy for Schwann cells migrating alone was smaller than when they migrated with fibroblasts. Typically, while tracking a group of cells the group entropy (order) was stable, however when fibroblasts are present on occasion large deviations were observed in the entropy. From observing the behaviour of cells, I found that interactions with fibroblasts lead to these temporary increases in entropy. This information suggests that adhesions between the Schwann cells within the cords are dynamic and cells are able to rearrange formation where necessary (see Fig. 4.38).

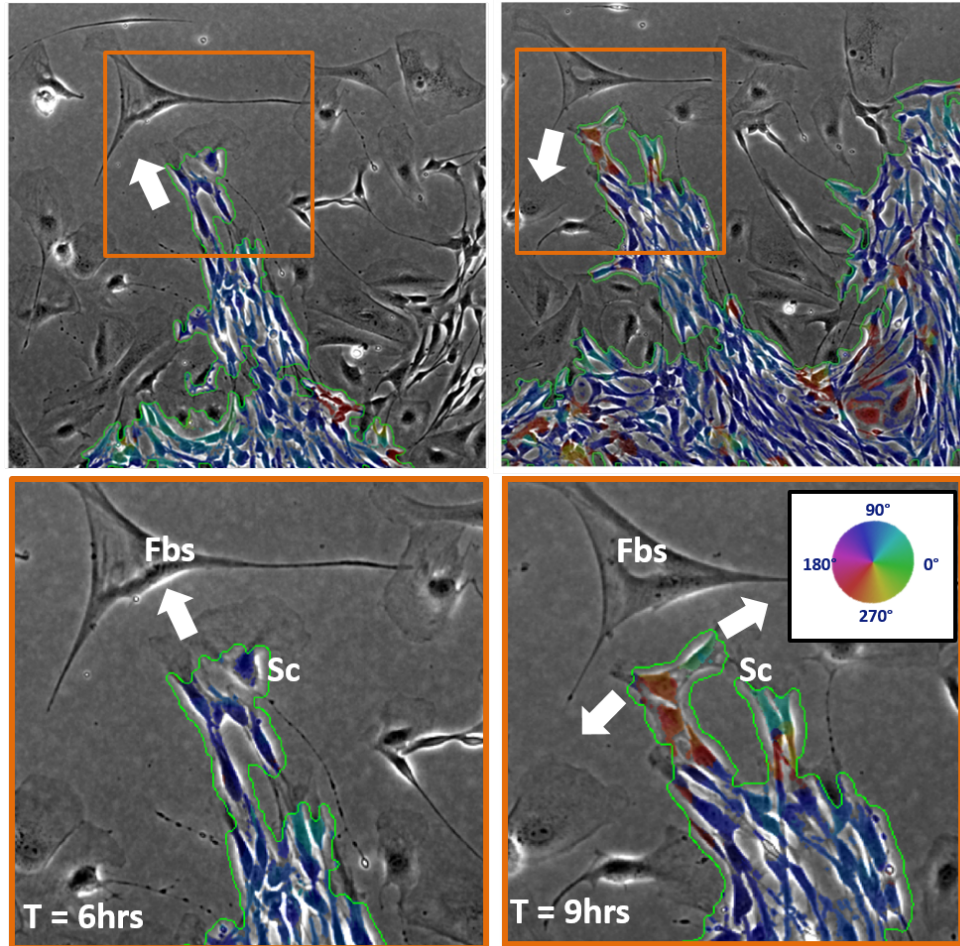


Figure 4.37: Schwann cells coming into contact with fibroblasts undergo a repulsive contact inhibition interaction. Using the optic flow, a colour map of direction can be used to aid understanding of cell behaviour from cell-cell interactions. White arrows show direction of motion (also indicated by overlaid direction field represented in colour). After interaction with a fibroblasts, Schwann cells nearest the fibroblast change direction and start moving to the left (orange cells) and right (green cells). Follower cells at 1-2 cell radii also change direction to follow the re-aligned leading cells. However, cells further back have maintained their heading.

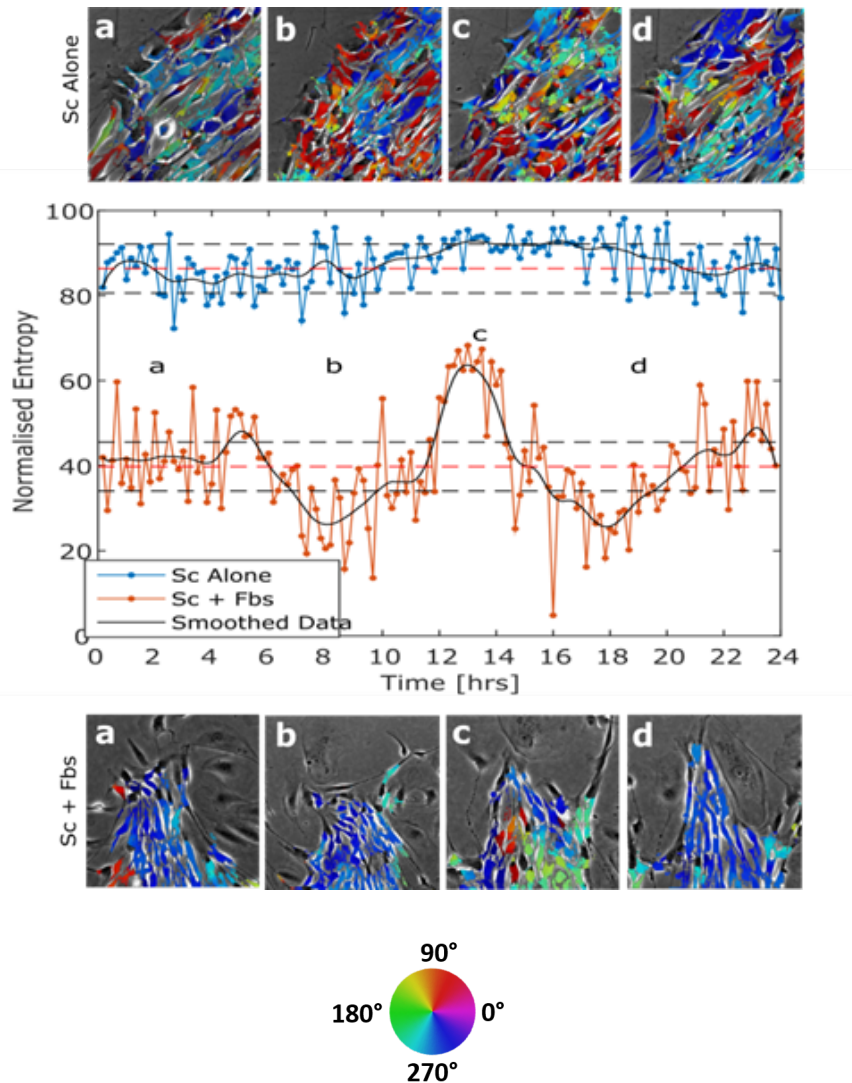


Figure 4.38: The entropy of the direction field of a group of cells can be used as a readout of local order. When Schwann cells migrate alone, the individual cell motion is uncorrelated, even on a local scale (i.e. nearest neighbour cells). This indicated that cell motion is predominately independent and cells do not interact in a way which results in correlated motion. Examples of a tracked group of cells migrating (alone) are shown in the top figure (a-d). The letters (a-d) corresponds to those in the middle figure, indicating the time-point the directional entropy of the group (blue line). The high entropy reflects high disorder in direction, however the variance in the entropy overtime is relatively small. This contrasts with the behaviour of Schwann cells when migrating in cords in the presence of fibroblasts. As can be seen in the bottom figure, when Schwann cells migrate in the presence of fibroblasts, neighbouring cells.

4.4 Summary and Conclusion

Fibroblasts have a clear impact on the manner in which Schwann cells migrate. When migrating alone in the migration assay setting, Schwann cells migrate out, spreading out from the initial blob symmetrically. Cells do not form long-term adhesions, and independent cells are often seen. However, when fibroblasts are also present in the migration assay, Schwann cells form cords and move together in an apparently cohesive manner. Using optic flow, I extracted velocity field from the live cell imaging data which allowed me to quantitatively describe the qualitative features observed. Using quantitative metrics to measure different features of collective cell migration *in vitro*, means the same metrics can be used *in silico* to parametrise the computational model.

Fibroblasts increase the global and local order of Schwann cells, meaning that the direction of motion at any given time is more similar between neighbouring cells and the whole ensemble when fibroblasts are present. I found that in the migration assay fibroblasts decrease the mean Schwann cell migration speed, but low local entropy (high local directional coherence) was correlated with higher migration speed. Thus in the cord like structures that form, where there is a higher coherence, large connected groups of cells move faster, while when alone, individual disparate cells move fast. Fibroblasts also increase the persistence of motion and directionality of migrating Schwann cells. Thus groups of Schwann cells have a larger displacement in a given time, despite the obstacles present by fibroblasts.

From the previous chapter I identified how fibroblasts affect Schwann cells, in this chapter I have quantitatively measured the effect of fibroblasts on collective migration. Together, this data suggest that fibroblasts play a dual role, they provide guidance cues via CIL, but also change the behaviour of Schwann cells, resulting in increased

N-cadherin at cell-cell junctions and thus cell-cell adhesions being maintained for longer. This results in Schwann cells migrating in cord like structures when in the migration assay. However, it is not clear how the impact of fibroblasts on individual Schwann cells results in the change in migration behaviour. Numerous questions arise which are not trivial to test experimentally, for example; would structural obstacles which have no effect on homotypic cell-cell interactions also results in cord formation or would increase adhesion alone be sufficient for cord formation. In the following chapter I describe my agent based model which uses features I have identified and to better understand how the phenotypes observed act to give rise to features of collective migration.

5

Computational Modelling

5.1 Rationale

Classically, collective motion of cells has been primarily studied by experimentalists who focused on collecting and reporting phenomenological observations. However, more recently some computational models have been developed which have been successfully used in conjunction with biological experiments to help elucidate the complex behaviour of collective migration. Computational models are particularly useful when the dynamics of a system cannot be elucidated by logic or simple reasoning alone. This is often the case even when the number of elements or reactions is small.

As computational and mathematical models can be used to represent, evaluate and analyse the current knowledge in the field, they can complement the traditional empirical and experimental approaches to research. They can be used as a tool for hypothesis generation and facilitate virtual experimentation when performing the actual experiment is too impractical or expensive. Simulations can also be used to make predictions about the outcome of particular parameter sets and initial conditions in real experiments.

Computational and mathematical methods can be top-down or bottom-up. Top-down methods start with data and try to make inferences about the underlying mechanisms or rules which lead to that data. Bottom-up models start with mechanisms of behaviour (data-driven hypotheses) and attempt to replicate (with computational analogues of those mechanisms) the observed data. Therefore, bottom-up modelling is useful when an abstract computational representation of a system is required. Whichever method or approach is used the models must generate explicit, testable hypotheses which can be validated with empirical data. Therefore, it is important to combine computational approaches with suitable quantitative measures which can be applied to both the empirical and theoretical data.

5.1.1 Modelling Approached in Collective Cell Migration

Emergent behaviour is the emergence of patterns or order in a system which results from relationships between elements of the system rather than an overarching rule. The phrase “the whole is greater than the sum of its parts” is often used to describe this phenomenon. Collective migration is an example of this, the emergent behaviour at the population level results from the way a cell behaves in response to its environment and cell-cell interactions. Predictive models can be formulated using a limited number

of simple, universal biological mechanisms.

Cells are complex; within a cell's cytoplasm, there are numerous functional and structural elements, each of which is involved in vital tasks. These components interact, allowing cells to respond in a myriad of ways to their environment and other cells. Furthermore, cells are not isolated, they communicate and interact with other cells and the environment via many signalling mechanisms. However, even the most detailed and careful study of the properties and mechanisms of a single cell and its signalling networks cannot predict the resulting behavioural dynamics at the multi-cell level. Thus, to study collective migration, cells behaviour is evaluated, rather than cell signalling networks, allowing the complexity of the problem to be significantly reduced. At the cell level (as opposed to sub-cellular level), cells have a limited number of behaviours and interactions. They can grow, divide, die, adhere to each other, repel each other, change shape, polarise, differentiate, create and remodel extracellular material, and migrate in response to signals in their environment. By making assumptions and using these simpler features, computational models can be built. Finite element and immersed boundary models allow for detailed geometrical representation of the shapes, surface properties and forces of cells, at much higher computational cost per cell. In these environments, one only needs to specify high-level parameters (rather than cell signalling networks); agent interactions, agent properties and how these properties change over time

Computational cell migration models have been typically formed using either continuum or discrete approaches. Continuum models describe features at the population-scale, while discrete models (also known as individual cell- or agent-based models) describe features at the cell length scale. Continuum models are derived from a deterministic, top-down perspective, where the dynamics of a large population of cells is described regarding locally averaged properties and have been used to model wound

healing, adhesion and chemotaxis, (Keller and Segel 1971; Sherratt and Murray 1990; Armstrong, Painter, and Sherratt 2006; Maini, McElwain, and Leavesley 2004). A population-scale property is a property which results from the behaviour of a large group of cells, a (soft) analogy to physics would be an extensive property. An example of this is the speed at which a population of cells migrates collectively is a population-scale property. To derive these models constitutive equations, obtained by analogy with well-established physical laws or intuition are used to describe how population features depend on the system variables (Sherratt and Murray 1990). However, because of this, the relationship between the resulting macroscopic continuum-based description and the microscopic cell-scale processes is not always clear. Continuum models are usually based on the inherent assumption that statistical correlations between cells are negligible, i.e. a mean-field assumption is made. However, a key aspect of the definition of collective cell migration is the correlated behaviour of local cells. This is observed during collective cell migration where correlations in the cell velocities are driven in large part by mechanical interactions (Serra-Picamal et al. 2012).

A population can also be described at smaller length scales, the cell-scale, and consider the properties attributed to a single cell. For example, the polarisation, speed, or "state" of an individual cell within a population of motile cells. The behaviour of a population is then determined by interactions between cells (via signals or mechanical forces), which in turn leads to strong cell-cell correlations. Discrete models use these ideas as a framework to model population behaviour. In contrast to continuum-based models; discrete models treat cells as discrete entities. In these models, key behaviours and interactions are abstracted from real biological processes, represent them as computational agents and rule-based interactions and then run simulations to observe the emergent phenomena. They are particularly useful in answering questions concerning the dependence of emergent properties on specific agent behaviours or environmental

perturbations. There are various cell simulation models and these trade off the level of detail per cell against the number of cells per simulation.

These models are often referred to as agent-based models (ABMs). At the cell scale, each variable in the ABMs corresponds to the centre of mass position of an individual cell; although, for finer-scale descriptions, variables can instead correspond to sub-elements of a cell. Agent-based simulations produce cell-resolution time series results, which can then be compared against experimental results through the selection of relevant characteristic metrics.

The positions of cells can either be restricted to specific lattice sites (lattice-based models; for example cellular automata) or be off-lattice (cells occupy positions in continuous space). Cellular automata represent cells as single, fixed lattice points. These models allow for the largest simulations but are limited in the possible cell movements and interactions. In lattice-based models where a cell only spans a single lattice site, cell movement is captured by transition probabilities (this being the probability of a cell jumping from one site to another). Transition probabilities are used to incorporate biological rules into the model. For example, by making transition probabilities dependent on whether a particular site is already occupied cell volume exclusion is captured; cell adhesion is incorporated by assuming that the probability of a cell vacating a site is lower if another adherent cell occupies a neighbouring site (Khain et al. 2009; Simpson et al. 2010)).

The lattice approach can only provide a phenomenological description of mechanical interactions between cells and, the coarseness of the lattice spacing prevents it from realistically capturing local correlations in the relative positions and velocities of neighbouring cells. To surmount these issues, the model can be changed so that a cell spans multiple lattice points (such as in the Cellular Potts Model (Chen et al. 2007)), where

alterations in cell size, shape or location are a consequence of these interactions. Alternatively, off-lattice models can be used, and in general, an off-lattice approach offers a better description of the underlying correlations (Sepúlveda et al. 2013). In off-lattice models, model variables can correspond to the positions of individual cells (treated as point masses) and move under interaction rules (Drasdo, Kree, and McCaskill 1995; “Many-body theory of chemotactic cell-cell interactions”; Pathmanathan et al. 2009; Szabó et al. 2006). Positions of cells are dictated by either stochastic (where stochasticity is used to capture random cell movement) or ordinary differential equations. Terms of the equation incorporate inherent motion and motion resulting from interactions with other cells or the environment. The key advantage of an off-lattice model is that this approach provides a way to more realistically capture interactions between cells than a lattice-based model.

5.1.2 Agent Based Models

Agent-based models are computational simulations of a group of agents and their interactions. In these systems, local interactions can result in interesting global-level (emergent) behaviour such as cooperation, segregation, or swarming. Agent-based modelling uses a rule-based, discrete-event and discrete-time methodology, where individual components (“agents”) interact with one another based on behaviour rules and “physical” laws of the system. The aim of this kind of simulation is to create populations of agents and simulate their actions and interactions in a “virtual world”, and thus, generate a *in silico* experimental model. By comparing the quantitative readouts of the dynamics of the model with empirical data, the correctness of the initial hypotheses (the rules/interactions) which underpin the ABM can be tested.

The essence of ABMs is that, instead of describing global, average dynamics, the

rules which lead to this phenomenon are identified. Cell interaction or cell migration experiments are a very natural system to translate into an agent-based model, as the autonomous agents are an obvious computational analogue of simplified cells and interaction rules are analogous to high-level cell signalling. This representation provides an intuitive link between the laboratory-derived basic mechanistic knowledge and the rules of the ABM. The benefit of using cell-level ABMs is that using abstract or simplified behaviour rules; they can be used to produce very familiar dynamics, and in so doing provide vital insights into the essential characteristics of a process. They are suitable for the study and analysis of subjects which involve decentralised decision making, local-interactions, self-organization, and emergence in simulated systems.

ABMs incorporate a number of elements which make them suitable for modelling cell migration. First and foremost, the rule-based structure, which can be based on direct observations from experimental work. Furthermore, the spatial nature of ABMs means agents can be governed by rules which are determined by its immediate environment, although non-local cues can also be built into the model. Additionally, ABMs are modular, meaning new aspects can be included via the introduction of new agent-types, by the modification of existing agent rules, or altering the environment without needing to rebuild the entire model. The emphasis on behaviour driven by local interactions and cues resembles closely the mechanisms of stimulus and response observed in biology.

ABMs can generate emergent properties as they cause dynamics in the system which could not have been inferred from analysis of the rules of the agents alone. Such emergent phenomena are global behaviours that are not derivable from the fundamental rules. Often, biological mechanisms are to some degree always incompletely known. However ABMs can be formed in the absence of full knowledge of the system, and it is

always advantageous to keep the rules as simple and testable as possible, even at the expense of some detail. Hypothesis testing via ABM provides qualitative verification of possible outcomes given incomplete knowledge.

The abundance of detail that ABMs can incorporate makes this methodology particularly suitable for the simulation of biological systems, where it is not easy or accurate to reduce the behaviour and heterogeneity of the interacting components to some stylised or simple mechanism. To construct an agent-based model you must specify; 1) a set of autonomous agents, 2) interaction rules which govern the behaviour of agents, 3) a simulated environment in which the agents live. Agents interact among themselves and with their shared environment. The simulated environment contains all other elements, for example; resources, other passive objects, as well as global properties and topographies. Finally, there must be a simulation infrastructure. This is the way in which the simulation is implemented. The infrastructure is responsible for the time advance, for visualisation, as well as for generating and exporting data. In principle, the infrastructure should not affect the outcome. However, technical aspects such as update-method (i.e. synchronous or asynchronous), and the order in which agents are updated can have non-trivial effects, and thus must be considered.

5.1.3 ABMs in Emergent Behaviour

In 1986, Reynolds proposed a distributed behavioural agent based model to describe coordinated animal motion, for example flocking in birds or shoaling behaviour in fish (Reynolds 1987). He named the generic simulated flocking creatures *Boids*, (a shortened form of "bird-oid object", and its pronunciation is akin to "bird" in a stereotypical New York accent). This model is not only the inspiration for many flocking and swarm simulations, but is also a powerful example of how simple, local interaction rules

can result in self-organisation and emergent behaviour. The complexity of this model arises from the interaction of individual agents (the boids) following a set of simple rules defined by the actuator. Reynolds basic flocking model consisted of three simple steering behaviours; i) separation, ii) alignment, iii) cohesion.

Table 5.1: Behaviours of the Boids Model

Behaviour	Description
Separation	Steering to avoid crowding local flock mates
Alignment	Steering towards the average heading of local flock mates
Cohesion	Steer to move toward the average position of local flock mates

The separation behaviour represents the boid's need of a personal space and is a mathematical approximation of the collision avoidance steering behaviour. Alignment represents the boid's wish to move with the same velocity as its flock mates. Finally, cohesion represents the boid's wish to be a member of the flock, rather than wandering off alone.

In 1995, Vicsek and his colleagues introduced a simple physical model to explore the emergent behaviour and order of n in a self-driven particles system (Vicsek et al. 1995). They proposed a simple model system to study the parameter space of a two-dimensional boids model. They showed that above a critical density, there is a transition to an ordered moving state. In this simple model the position of a particle is given by

$$\mathbf{x}_i(t+1) = \mathbf{x}_i(t) + \mathbf{v}_i(t) \cdot \Delta t \quad (5.1)$$

$$\theta(t+1) = \langle \theta(t) \rangle_r + \Delta \theta \quad (5.2)$$

where $x(t)$ is position, $v(t)$ is velocity, $\theta(t)$ represents heading (direction). The speed

$v_i(t)$ in equation 5.1 has constant module v_0 , and the direction each particle (boid) takes results from an average over directions of neighbouring boids (within a given radius) and a noise term. $\langle\theta(t)\rangle_r$ denotes the average direction of the velocities of particles (including particle i) which are within a circle of radius r centred on the given particle. The average direction is given by the angle $\arctan[\langle\sin(\theta(t))\rangle_r/\langle\cos(\theta(t))\rangle_r]$. In equation 5.2, the term $\Delta\theta$ represents noise and is a random number drawn with a uniform probability from the interval $[-\eta/2, \eta/2]$. In this model there are three free parameters for a given system size: noise (η), density ($\rho = N/L$) where N is the number of agents and L is the area, and speed (v), where v is the distance a particle makes between two time points. Using this model they found that when the density of all agents is large enough and the system noise is small, all agents tend to move in the same spontaneously selected direction collectively. While this realization is elegant because of its simplicity, it does not capture key elements of the biological realities. Particularly absent are cell-cell interactions which we know occur a term to account for cell volume and the absolute value of the velocities do not reflect biological systems.

Since then, many agent based models have been used to study emergent behaviour from traffic flow (“Agent-based modeling: Methods and techniques for simulating human systems”), ant behaviour (Pratt et al. 2005), human behaviour (Haeger2015b), tumour formation (Zhang et al. 2009) and cell migration (Woods et al. 2014). Variations on types interaction rules, environment, and self-propulsion have been used to explore behaviours Using these ideas and models as inspiration I ask how the impact of fibroblasts on Schwann cell can lead to emergent collective migration. By combining features measured from the data and quantitative metrics from the data I elucidate the relationship between features and their importance in the cord like collective migration. Concerning cell migration models, my model differs in that the agents have memories and can be in different states, and the agent population is heterotypic, which leads

to local spatial cues changing agent behaviour (as fibroblasts change the behaviour of Schwann cell via direct cell contact).

5.2 Collective Cell Migration Agent Based Model - CCMABM

In this section, I introduce my agent-based model (ABM) of cell migration and interactions. The model is intended to be able to capture the motion of cells that interact through physical forces such as cell-cell adhesion and CIL, but simple enough so that it is computationally tractable to simulate and fit parameters. I first introduce the model concepts and mathematics of the model and then go on to describe the methods to implement these ideas. To build the model I describe; (1) the environment in which agents live, (2) the agents, (3) the motion rules of agents, (4) the interaction rules of agents. In brief, cells are represented as agents which live in a 2D simulated space. Terms are included to account for cell volume, for self-propulsion, local adhesion based forces and contact inhibition of locomotion based forces. The mathematical form of cell behaviours; self-propulsion, adhesion, contact inhibition and volume exclusion are abstracted from the biological definition.

1. Environment

Agents (simulated cells) were initialized in the centre of the environment, (square domain of size L) in a circular template to best mimic the blob in the *in vitro* experiments. The number of Schwann cell agents was set to obtain a close to fully packed coverage of the entire Schwann cell initiation simulation area. The number of fibroblast agents relative to Schwann cell agents was a variable, however, for simulations where this was not being explicitly studied, the number of fibroblasts agents was determined based on *in vitro* experiments. Fibroblast agents were initialised on an equally spaced grid, and only outside the Schwann cell initiation area. To simulate the random distribution of fibroblasts observed in *in vitro* experiments, the position of fibroblast agents were then perturbed, with the rule that if any fibroblasts overlapped each other, one of the fibroblast agents would be removed, ensuring no overlap of fibroblasts.

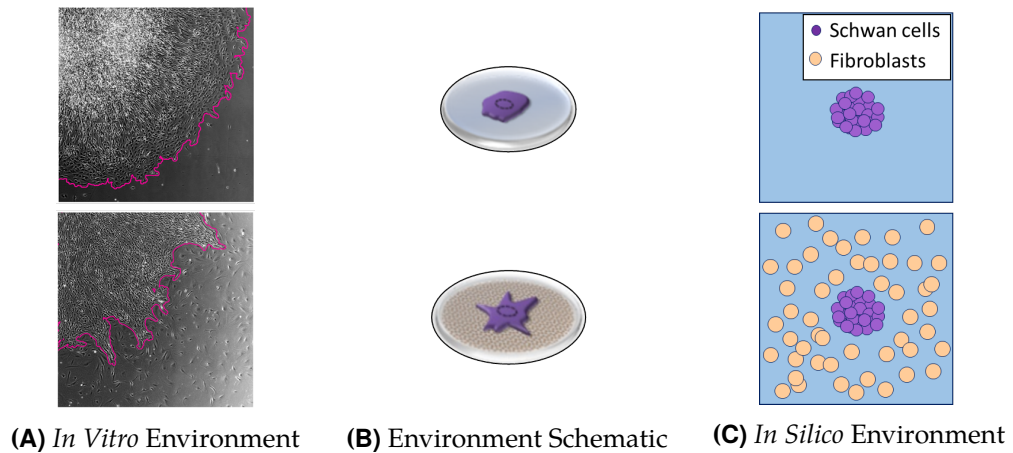


Figure 5.1: Agents are modelled in a 2D space. The initial spatial location of cells in the model (C) is designed to recapitulate *in vitro* experiments (A), a dense group of cells which can migrate out into either an empty area (Sc alone) or an area in which fibroblasts are present (Sc + fbs). (C) Schwann agents are shown as purple circles and fibroblast agents are shown as orange circles.

2. Agents

Each cell (either Schwann cells or fibroblast) is an agent and is modelled as an interacting self-propelled point particle which at each instance of simulated time have; a position, a velocity and a phenotype state. Cells also have a set of radii which describe the cell size and interaction zones for adhesion and repulsion.

Interaction zones are the areas within which an agent can sense and thus interact with other agents. These are; the cell radius, r_c , the separation radius, r_{sep} , the adhesion radius, r_{adh} , and the fibroblasts perception radius, $r_{Sc/fbs}$. The cell radius, r_c denotes the size of the Schwann cell or fibroblasts, the fibroblasts perception radius denotes the distance at which Schwann cells and fibroblasts can interact and $r_{Sc/fbs} = r_{Sc} + r_{fbs}$, where r_{fbs} is the radius of the fibroblast agent and r_{Sc} is radius of the Schwann cell agent. The cell separation radius (see the orange circle in Fig. 5.2A) denotes the upper bound of the region of any form of cell contact induced separation; volume exclusion or contact inhibition of locomotion. The adhesion radius, r_{adh} , (see the purple circle in Fig. 5.2A) denotes the upper bound of the spatial region of cell-cell adhesion. The adhesion radius is a function of the cell radius and the adhesion zone factor, ϕ_{adh} , which in turn is a function of cell behaviour-state, see Fig. 5.2B. This is inspired by biological observations which show cells have incompressible bodies (the cell cytoplasm is an incompressible fluid) and can form adhesions at protrusions which extend beyond the main cell body (DeMali and Burridge 2003; Ridley et al. 2003; Le Clainche and Carlier 2008), see Fig. 5.6. Formally an agent can be described by, $q \in Q$,

$$\text{Agent} = \{q | q = \langle \mathbf{x}, \mathbf{v}, r, s \rangle\} \quad (5.3)$$

where $\mathbf{x} \in A$, is position, $\mathbf{v} \in A$ is velocity, and A is 2D Euclidean space, $r = \langle r_c, r_{sep}, r_{adh}, r_{fbs} \rangle$, are the cell size, separation, adhesion and fibroblasts repul-

sion radii, $s = \{s^0, s^+\}$ the phenotype state.

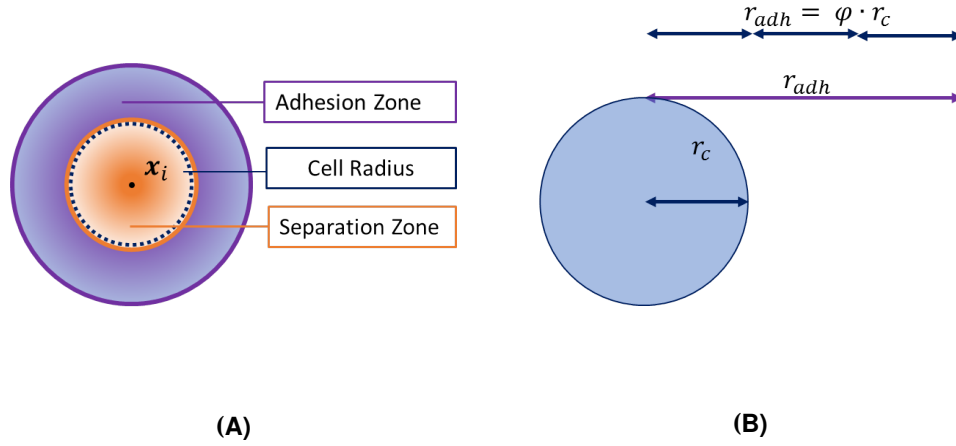


Figure 5.2: Cells (agents) are modelled as point particles with a cell radius, r_c , separation radius, r_{sep} , and a radius which describes the region of cell-cell adhesion, r_{adh} , which is a function of the cell radius/separation and the adhesion zone factor, ϕ , which is a function of cell state.

Direct cell-cell interactions between Schwann cells and fibroblasts results in phenotypic changes occurring in the Schwann cells. Therefore, I include this change in phenotype by describing agents being in one of two states; high or normal adhesion denoted as s^+ and s^0 respectively. A state change is a result of local interactions (see point 4) between a Schwann cell-agent and a fibroblast-agent. The state change is instantaneous and impacts the state of the cell at the incidental update. Cells either stay in a normal adhesion state (blue state (s^0) in Fig. 5.3) or transition to the high adhesive state (s^+) following interaction with a fibroblast (red state in Fig. 5.3).

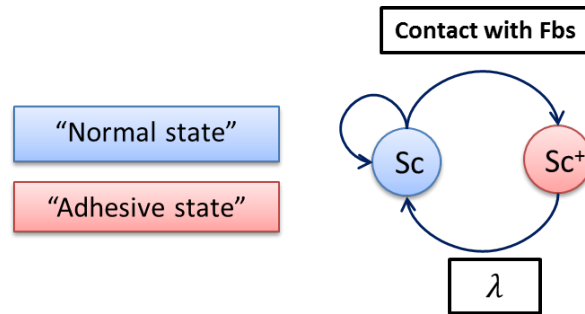


Figure 5.3: Schwann cells are represented as agents which can be in one of two states; normal (blue) or adhesive (red). The state change occurs if a Schwann cell agent comes within the interaction radius of a fibroblasts agent. The state change is instantaneous. The adhesive state decays back to the normal state with a rate factor λ , which was estimated from *in vitro* experiments.

Schwann cells which have come into contact with fibroblasts exhibit a striking change in morphology, becoming elongated in contrast to their usual more rounded shape. This change is observed both in the cell mono/co-culture assays and in the migration assay. To integrate this idea into the model I use different interaction radii for cells which are in normal, s^0 , and high, s^+ state. Agents in state s^0 have a larger separation radius and a smaller adhesion radius than those in s^+ , as is shown in figure 5.4.

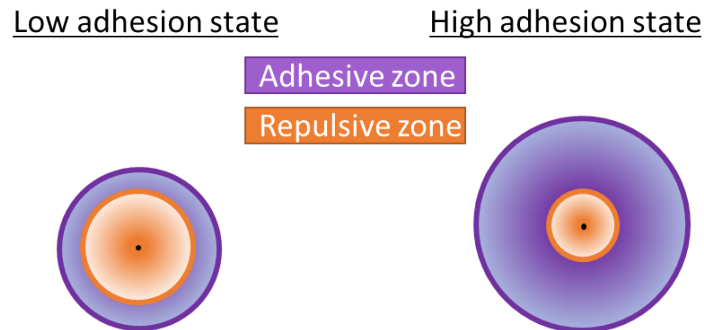


Figure 5.4: Schwann cells are represented as agents which can be in one of two states; normal or adhesive. Evidence from *in vitro* experiments (see Chapter 3) show morphological changes as a result of fibroblast/Schwann cell interactions. Agents in state s^0 have a larger separation radius and a smaller adhesion radius than those in s^+ .

These have been extracted directly from experimental data as shown in Fig. 5.6. To estimate the distance at which cells feel a separation force I measured the distance between nearest neighbour nuclei *in vitro* for Schwann cells alone (yellow lines in Fig. 5.5A) and Schwann cells + fibroblasts (yellow lines in Fig. 5.5C). To estimate the adhesion radius I measured the protrusions *in vitro* for Schwann cells alone (white lines in Fig. 5.5A) and Schwann cells + fibroblasts (white lines in Fig. 5.5C).

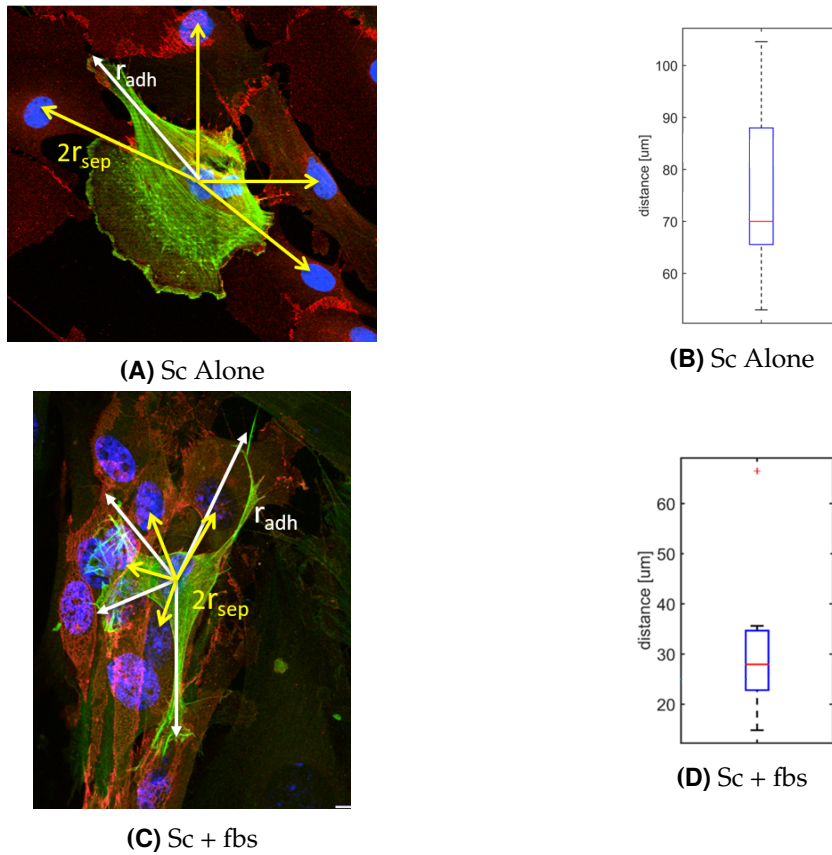


Figure 5.5: Representative images of (A) Schwann cells alone and (B) Schwann cells + fibroblasts labelled with LifeAct (actin label) to show the actin cytoskeleton (green), N-cadherin (red), and cell nuclei are stained with Dapi (blue). Biological observations which show cell have an "incompressible" body and can form adhesions at protrusions which extend beyond the main cell body. The adhesion and separation radii are estimated by measuring the distance between nuclei (yellow lines) and the adhesion radius is estimated by measuring protrusions (white lines), $n=100$ cells from 3 different experiments were measured in this way.

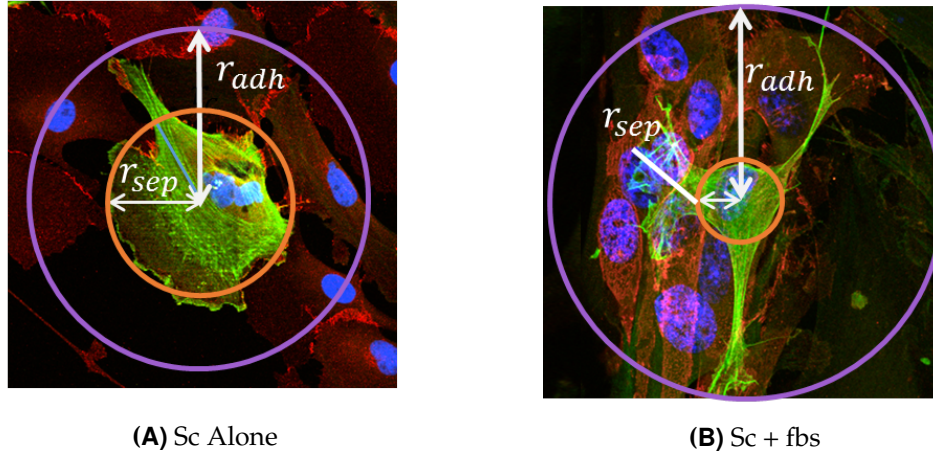


Figure 5.6: Representative images of (A) Schwann cells alone and (B) Schwann cells + fibroblasts labelled with LifeAct to show the actin cytoskeleton (green), N-cadherin (red), and cell nuclei are stained with Dapi (blue). Orange circles represent the estimated repulsion radius r_{sep} and purple circles represent the adhesion radius r_{adh} .

I assume that only the cells position $\mathbf{x}$, velocity $\mathbf{v}$ and state s can change through time, while all other parameters stay constant. For simplicity, all agents in a given state have the same radii, which was estimated from the mean value of 50 cells measured from three separate experiments. If a larger sample of cells had been measured, the size of each agent could be randomly selected from a distribution representative of the experimental data.

3. Locomotion rules

Agent motion is described using Newtonian dynamics. For a particle in a viscous fluid, according to Newtonian dynamics, the particle accelerates in response to forces which act on it, and the deterministic equation governing motion is:

$$m_i \frac{d^2 \mathbf{x}_i}{dt^2} = -\gamma \mathbf{x}_i + F_i^v + \sum_{j,j \neq i}^N F_{i,j} + F_v + \epsilon_i \quad (5.4)$$

Where m_i is the mass of agent i , F_i^v is the self-propulsion force, $\sum_{j,j \neq i}^N F_{i,j}$ is the sum of external forces, F_v is the viscous force term and ϵ is a Gaussian noise term. Due to the small size and slow speed of motion of cells, the inertial term (LHS) in 5.4 can be neglected when considering cell motility. Briefly, by considering the relative importance of the inertial and viscous terms in the equation Equation 5.4, the inertial term can be dropped as it is much smaller than the viscous term. This is shown by considering the Reynolds number, which is the ratio of the inertial to viscous forces,

$$Re = \frac{F_{inertial}}{F_{viscous}} = \frac{\rho a \nu}{\eta} \quad (5.5)$$

where a is the size, ρ is the density, ν is the speed and η is the viscosity. For cells of the order of $\approx 10\mu$ and velocity of order $20\mu s^{-1}$, $Re \approx 10^{-4}$. For an in depth discussion see (Purcell 1977), this means the cell velocity is proportional to external forces and its self-propulsion. The external forces comprise interactions between cells and the environment,

$$\frac{d\mathbf{x}_i}{dt} = \gamma \left(\eta \sum_{j,j \neq i}^N F_{i,j} + v_i \mathbf{n}_i + \epsilon_i \right) \quad (5.6)$$

where $\mathbf{x}_i$ is the position of cell i . The first term of the RHS of equation 5.6 describes the effect of external forces on the agent, where the factor η modulates the importance of the external forces. The second term describes the self-propulsion term. The vector

$\mathbf{n}_i$ is the cell heading unit vector, which changes in time according to,

$$\frac{d\theta_i}{dt} = \arg(\mathbf{v}_i) - \theta_i + \epsilon \quad (5.7)$$

where $\theta_i = \arg(n_i)$ is the angle of the heading vector n_i and $\arg(\mathbf{v}_i)$ is the angle of the actual velocity as influenced by inter-particle forces. Since the steering is imperfect, a noise term ϵ is also included. This is Von Mises noise with zero mean and concentration a function of cell state. By using equation 5.7, the effect of cell polarization and persistence in motion can be included. A polarised cell is more likely to continue in the direction it is currently moving. To account for this behaviour in my model, I describe a term which I call "stochastic directional persistence". This noise term is drawn from a Von Mises (circular normal) distribution, with the mean, μ , which is equal to the previous direction of motion and a concentration value, κ . The mean, μ , is a measure of location, and the distribution is clustered around this value. The concentration value, κ is a reciprocal measure of dispersion: $\frac{1}{\kappa}$ is analogous to σ^2). If κ is zero, the distribution is uniform, and for small κ it is close to uniform. However, if κ is large, the distribution becomes very concentrated around the angle μ . Therefore, for a greater value of κ the persistence in directionality is greater, since there is a higher probability of continuing in the same direction. In Fig. 5.7 I show examples of this movement update term and how different values of κ effect migration. I used root mean square displacement as a function of time to assess how the value of κ for a given step size and no external noise effects migration. As can be seen in Fig. 5.7D, when κ is equal to zero, the root mean square displacement (RMS) tends to the square root of N , as is expected for a random walk. For higher (example) values of κ the RMS is significantly higher than a random walk. Therefore I can use this term as a tunable noise term, which can encapsulate the idea of persistence in motion, based on previous motion (most recent motion only) from changing on the concentration parameter.

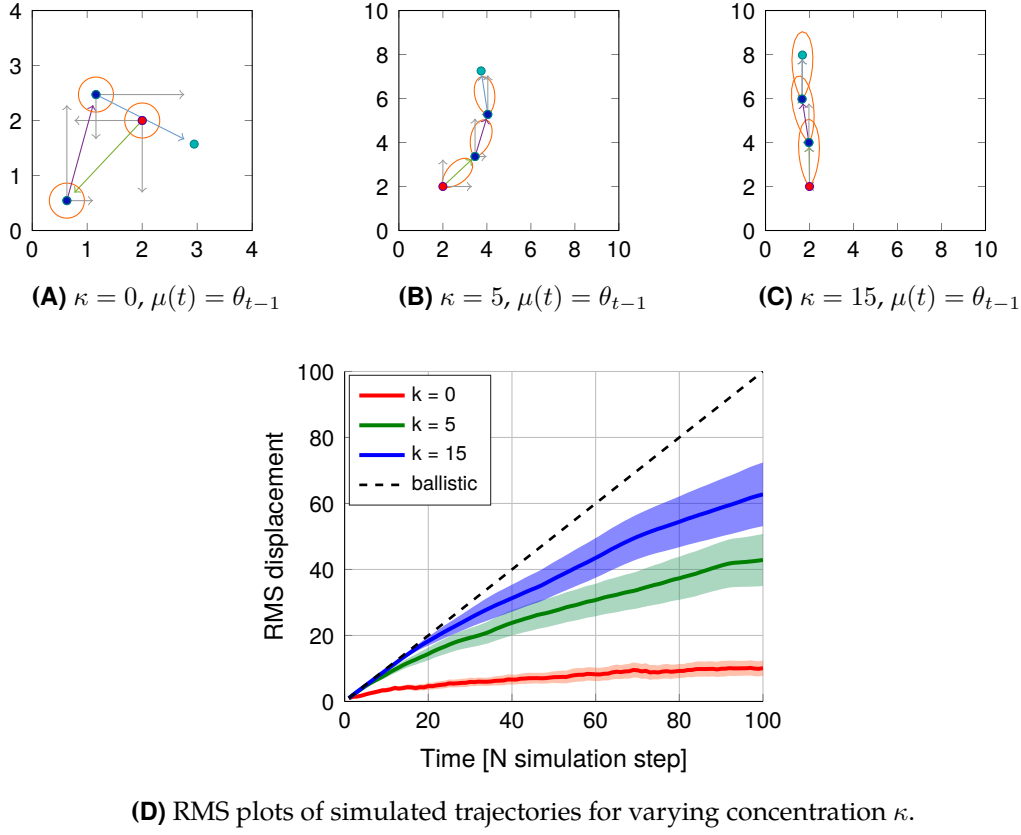


Figure 5.7: The simulated cell is shown in teal, with the previous locations shown in dark blue. The initial position is at (2,2) shown in red. Movement vectors are shown with arrow (green, purple, blue for $t = 1, 2, 3$ respectively), and the step size is equal in each example and for all time steps. Movement vector decomposed into x and y direction vectors are shown in light grey. The Von Mises probability distribution is $f(\mu, \kappa)$ is shown in orange. The distribution is concentrated (according to κ) around the mean μ , which is the direction of movement from the previous time step. The distribution is more concentrated for higher values of κ , resulting in smaller changes in direction between subsequent movement, and therefore more persistent motion. (D) RMS plots of simulated trajectories for varying concentration κ . As κ increases the motion moves away from diffusive to ballistic. This is used to model the persistent motion of cells.

This differs from the inclusion of solely a Gaussian white noise term, ϵ with zero mean and finite variance which is included in Brownian motion to account for stochastic

processes because it considers the role of persistent motion.

$$\frac{d\theta_i}{dt} = \epsilon \quad (5.8)$$

$$\langle \epsilon_i(t) \epsilon_j(t') \rangle = 0, \quad (5.9)$$

$$\langle \epsilon_i(t) \epsilon_j(t') \rangle = 2D\delta_{i,j}\delta(t - t') \quad (5.10)$$

4. Interaction Rules

When isolated, cells will move solely from self-propulsion, but when they are in the vicinity of other cells, their motion is influenced by interactions with their neighbours. The inter-cellular interactions are: 1) cell-cell adhesion, from N-cadherin, mediated adhesion, 2) repulsive interactions (hetero- and homo-typic). Each interaction produces a force that induces a change in velocity. The repulsive behaviour is used to a) introduce volume exclusion and b) contact inhibition of locomotion. The magnitude of the repulsive forces proportional to the distance to the agent it is interacting with, the closer an agent is, the larger the force applied. However, as this is a contact dependent effect, agents only have local knowledge and thus only agents within the separation radius experience this force. The adhesive behaviour is responsible for keeping agents together. Regarding forces, I consider adhesion as a force that moves agents in the direction of the centre of mass, x_{com} , of its neighbours which fall within the adhesion radius.

I model the cell-cell interaction forces with a distance dependent function, $F_{ij} = f(d_{ij})$, where d_{ij} is the distance between two agents, $|\mathbf{r}_i - \mathbf{r}_j|$. If the distance d_{ij} is less than the cell radius, r_{sep} , the force is repulsive, while if d_{ij} is greater than r_{sep} but less than the maximum radius of adhesion r_{adh} the force is attractive. If d_{ij} is greater than r_{adh} ,

there is no force between the cells.

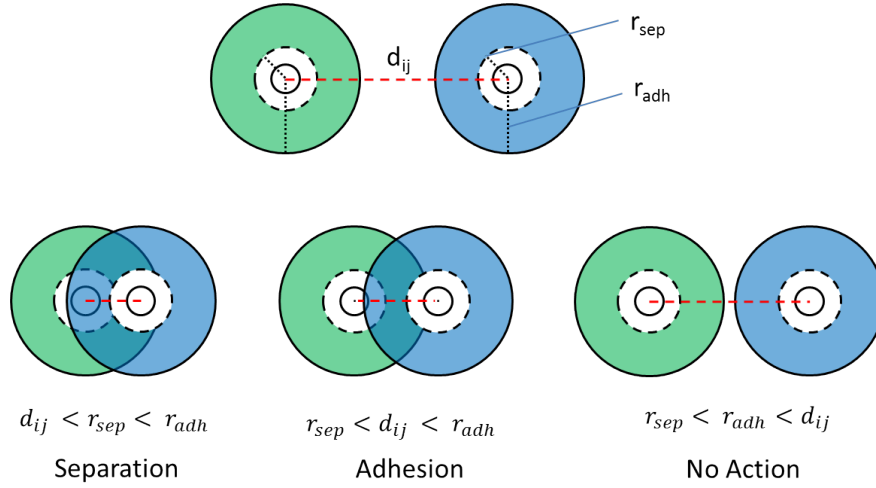


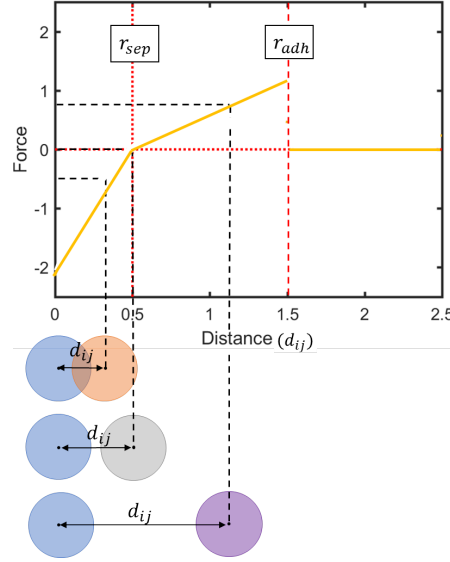
Figure 5.8: Top panel: the distance between two agents is evaluated by the distance between their actual point location (dashed line, d_{ij}). Bottom panels: examples of how initial inter-cellular distance d corresponds to different cell interactions. Left, if $d_{ij} \leq r_{sep}$, cells will exert a separation force on each other since their bodies overlap. On the contrary. Middle, if $r_{sep} \leq d_{ij} < r_{adh}$ cells will interact with adhesive forces. Finally, if $d_{ij} > r_{adh}$, cells are far enough apart that they do not interact.

To incorporate both shorter range repulsive/separation interactions and longer range adhesive interactions I used the piecewise linear function (see 5.11). The form of this model is shown in Fig. 5.9, which show that while the distance between two agents is less than the separation radius, the force is negative -short range repulsion- while when it is greater than the separation but less than the adhesion range, the force is

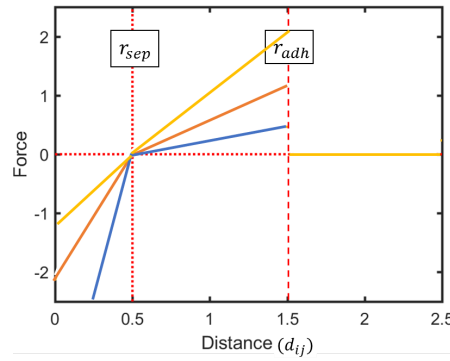
positive - longer range adhesion. This is formed using explicit boundary rules.

$$F(r_i, r_j) = \frac{r_i - r_j}{d_{ij}} \begin{cases} F_{sep} \frac{d_{ij} - r_{sep}}{r_{sep}} & \text{if } d_{ij} < r_{sep} \\ F_{adh} \frac{d_{ij} - r_{sep}}{r_{adh} - r_{sep}} & \text{if } r_{sep} < d_{ij} < r_{adh} \\ 0 & \text{if } d_{ij} > r_{adh} \end{cases} \quad (5.11)$$

I use a piecewise linear model to describe the interaction force for two main reasons; 1) the coefficients which control the strength of the interactions can be adjusted easily and the impact of this is transparent, 2) I do not have any evidence on the distance dependent force model of cell interactions, therefore I decided to choose the simplest model. The way in which cells interact with their neighbours depends on their "state" which is determined by their interaction with fibroblasts. To incorporate the change of behaviour as a function of state, the interaction strength coefficients are a function of state, $F_{sep}^i = F_{sep}(s)$, $F_{adh}^i = F_{adh}(s)$. This is clearer to implement in the piece wise model, where the two interactions are independent.



(A) Delineating interaction zones of the piece-wise linear model



(B) Effect of changing the gradient on the piece-wise linear model

Figure 5.9: (A) Piece-wise linear function which show the interaction force as a function of the distance between neighbouring cells. In both cases, cells with a separation distance d_{ij} less than the cell diameter induce a negative force, while those greater than a cell diameter and less than a threshold distance induce a positive force. (B) To alter the interaction strength in the linear piece-wise model the gradient coefficients in the separation (F_{sep}) and adhesion (F_{adh}) region can be altered.

5.3 Model Implementation

Finite Difference Equations

The mathematics which describe how agents move and interact have been set out above, and here I describe how these implemented as an *in silico* simulation. The continuous-time solution to the set of differential equations given by equations 5.6 and 5.7, can be approximated by a discrete-time sequence $[r_i; \theta_i] \approx [r(t_i), \theta(t_i)]$ that is the solution of the corresponding set of finite difference equations evaluated at regular time steps $t_k = kDt$, where Dt is a sufficiently small time step.

$$\frac{d\mathbf{r}}{dt} \rightarrow \frac{\mathbf{r}_k - \mathbf{r}_{k-1}}{\Delta t}, \quad (5.12)$$

$$\frac{d\theta}{dt} \rightarrow \frac{\theta_k - \theta_{k-1}}{\Delta t} \quad (5.13)$$

$$\mathbf{r}_k = \mathbf{r}_{k-1} + \Delta t \cdot \left(\eta \sum_{j,j \neq i}^N F_{i,j} + v_0 \mathbf{n}_k \right), \quad (5.14)$$

$$\theta_k = \theta_{k-1} + \Delta t \cdot (\arg(v_{k-1}) - \theta_{k-1}) + \frac{\epsilon}{\sqrt{\Delta t}} \quad (5.15)$$

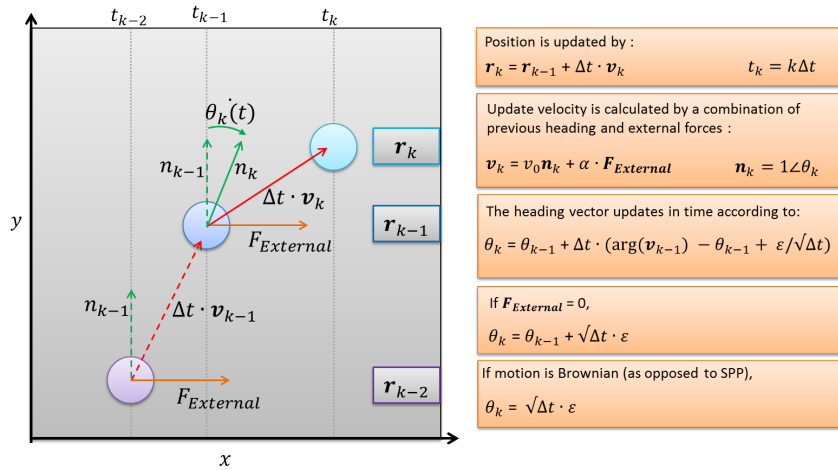


Figure 5.10: Schematic of how equations of motion are implemented in the *in silico* model.

Cell-Cell Interactions

As discussed in the introduction (see Fig. 1.2), it can be difficult to distinguish a contact inhibition of locomotion (CIL) mechanism from a purely physical phenomenon of volume exclusion. Analysis of CIL in vitro confirms that the mechanism of contact inhibition is different from the dynamics of an equal mass normal force rigid body collision. Cells occupy physical space, thus when they come into contact, they must either steer around, climb over or repel away from each other. CIL is a repulsive interactions wherein the direction of movement - following cell-cell interaction - is steered away from the point of contact with another cell. Volume exclusion, however, prevents cells from encroaching on the space occupied by the other cell but permits the cells to move into any nearby space that is empty of other cells, i.e., there is no bias in the direction of movement away from the point of contact.

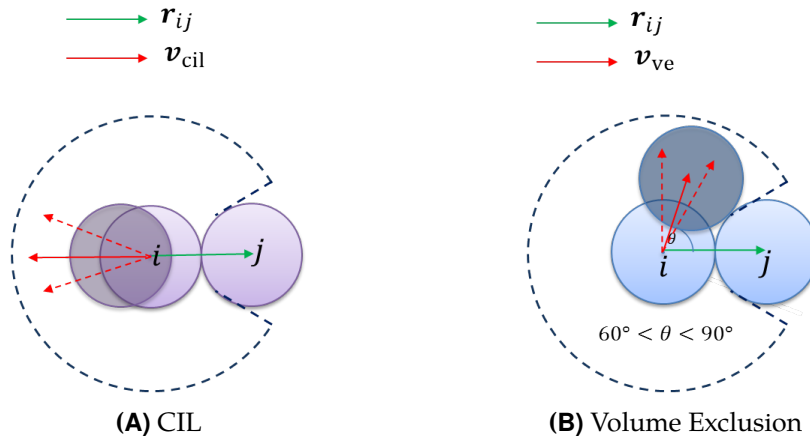


Figure 5.11: Schematics showing difference in repulsive vector from CIL and volume exclusion assuming a sparse environment where the agent j is stationary.

An attempted move is aborted if it would result in two individuals overlapping (i.e. if the centres of two cells would be closer than $2 \cdot r_{cell}$). Therefore, to describe contact inhibition locomotion, the force is modelled as a repulsive force which, for a agent i with respect to a neighbouring agent j , acts in the direction where the angle is drawn from a Von Mises distribution with μ given by the interaction vector $r_{ij} = r_j - r_i$ and the concentration parameter $\kappa = 10$. For volume exclusion the model is modified by decreasing the concentration parameter to simulate the addition of a re-polarisation force that acts in a randomly distributed direction at the free edge.

$$f(x|\mu, \kappa) = \frac{e^{\kappa \cos(x-\mu)}}{2\pi I_0(\kappa)} \quad (5.16)$$

where, $I_0(\kappa)$ is the modified Bessel function of order 0, μ and $1/\kappa$ are analogous to μ and σ^2 (the mean and variance) in the normal distribution. If κ is zero, the distribution is uniform, for small κ , it is close to uniform and if κ is large, the distribution is very concentrated about the angle μ .

Thus, the resulting repulsive velocity vector for an agent i interacting with an agent j is given below by equations 5.17 and 5.17, where f is the Von Mises distribution given in equation 5.16.

$$\mathbf{v}_{CIL} = F_{sep}(d_{ij}) \angle f(\arg(\mathbf{r}_{ij}), \kappa_{cil}) \quad (5.17)$$

$$\mathbf{v}_{ve} = F_{sep}(d_{ij}) \angle f(\arg(\mathbf{r}_{ij}), \kappa_{ve}) \quad (5.18)$$

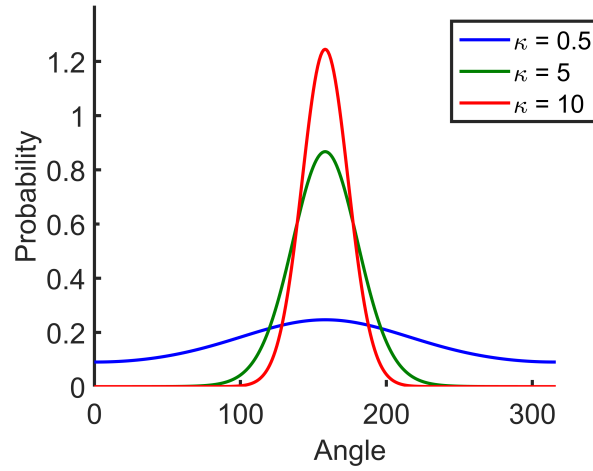


Figure 5.12: Von Mises Distributions with $\mu = 180$ and varying the concentration parameter κ . The distribution tends to the normal distribution at $\kappa \rightarrow 0$ and becomes more peaked for larger κ . This is used to modulate the repulsion angle, a larger κ is used if the deviation from the simple repulsion angle should be small (as in CIL) or a smaller κ is used if the deviation should be larger as in volume exclusion.

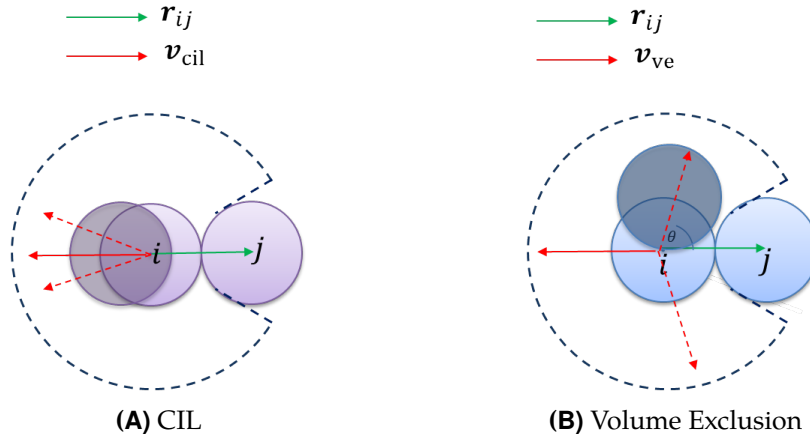


Figure 5.13: Schematics showing difference in repulsive vector using the Von Mises method for (A) CIL and (B) volume exclusions. The solid red angle shows the mean of the distribution and the dashed red lines show the effective standard deviation from the mean. A smaller concentration value (larger standard deviation) results in a larger allowed area into which the cell moves from the repulsive interaction. The effect of this is that volume exclusion is a less directional effect than CIL.

Synchronous vs. Asynchronous Updating

In the implementation of agent-based models, the order in which the positions of the agents are updated must be specified. There are different ways you can define the order, and the method used for updating can have implications on the results of the model. The simplest approach for agent-based models is synchronous updating. In this system, all agent movement or changes are calculated for time step 1 using the information of all the agents in time step $t - 1$. In this method the order in which agents are updated is not important as only the information from the previous time step is used. In the majority of real systems, information is not updated in a synchronous manner. Rather, it is more likely to be a continuous process where some agents make decision earlier than others. This is called asynchronous updating, and the order in which the agents are updated now becomes important. For example, if you are updating agent i and agent j , since the outcome of decisions made by agent j will depend on the position of agent i , and visa versa, you can derive different outcomes when first agent i is updated and then agent j , or the other way around. One can use a fixed order of updating. For example, you can update the agents 1 to N always in the same order. This can lead to artefacts, which is problematic, so a common approach is random asynchronous updating. Each time step, agents are updated in a random order, so on average, each agent gets equal opportunities. Finally, we can define a sequence based on some property of the agents. For example, agents with few neighbours could be prioritised in the update sequence. Using an asynchronous method for updating position allows for “impossible behaviour prevention. An impossible behaviour is one which in real life would be not allowed to occur, for example two agents moving through each other or an agent moving onto another where it should be immediately prevented via CIL. If the velocity update for a given agent results in an impossible behaviour we can either cancel this update outright or move it a fraction of its update and iteratively check if this behaviour is allowed.

5.4 Using the model

We used the model described in the previous section to understand how local interactions with fibroblasts leads to cord formation. I used the following framework for investigation:

1. Fit unknown parameters for Sc Alone simulations, using biological valid assumption to constrain the parameter space.
2. Explore the behaviour of the model under perturbations.
3. Assume that the underlying behaviour of Sc is maintained and find parameters of changed state cells
4. Use the model to explore how cords can form in collective cell migration.

Where possible I have used parameters which have been taken from *in vitro* experiments. Many parameters which could affect the emergent behaviour, such as the initial configuration, size of cells (Schwann cells and fibroblasts), or relative number of Schwann cell to fibroblasts are fixed from information from the biological experiments. Thus, the agents in state 1, no fibroblasts contact are larger, have a smaller adhesion radius, are more motile and have weaker adhesive force among each other. Agents in state 2, those which have come into contact with fibroblasts and not switched back to state 1, I assume that both short-range repulsion and the attractive force can act between all agents, i.e. like-unlike agents can interact as well as like-like agents. The parameters which were not informed directly from the data were inferred from the *in vitro* live cell imaging data using a grid search method (see Chapter 6).

Table 5.2: Parameters for Model

Parameter	Description	Value
r_{sc}	Radius of Schwann cell	25
r_{fbs}	Radius of fibroblast	50
$\phi_{s=1}$	Adhesion radius coefficient	$2r_{sc}$
$\phi_{s=2}$	Adhesion radius coefficient	$4r_{sc}$
r_{sep}	Separation radius	0.2
$v_{s=1}$	Speed	1.25
$v_{s=2}$	Speed	2
$\epsilon(s=1)$	noise	$f(0, \kappa = 0.5)$
$\epsilon(s=2)$	noise	$f(0, \kappa = 5)$
$\angle F_{CIL}$	CIL angle	$f(\theta_{ji}, \kappa = 0.5)$
$\angle F_{ve}$	VE angle	$f(\theta_{ji}, \kappa = 5)$

Simulation of Sc Alone under CIL and self-propulsion

I ran simulations of the process when the Schwann cells were alone. As an initial condition cells were initiated within a circle of radius, r , such that agents overlap, and all agents types were the same. Snapshots of simulation are shown in Fig. 5.14, cells migrating outward, recapitulating what is observed in the *in vitro experiments*.

To parametrize the model initially only the mean local entropy was used. This is because, as the model is not mathematically well-defined (i.e there is not an analytic solution), it has to be run many times (due to stochastic events), it is very expensive and difficult to optimise parameters. A grid optimisation method was chosen over gradient descent or other optimization methods. A grid search of reasonable parameters was explored (reasonable parameter bounds were based on biological understanding of the system) and for each grid coordinate the model was run 7 times, which was found to be sufficient in the output variance while still being computationally feasible. The optimal parameter was decided by finding the grid-coordinate which gave the minimum difference between the squared difference between the mean value of local

entropy between *in vitro* and *in silico* data. Simulation outputs were also examined by eye to ensure that parameters which gave a good fit also gave reasonable outputs of model behaviour.

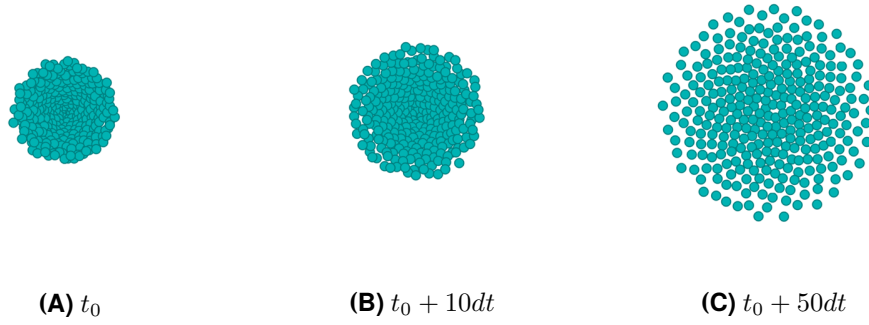


Figure 5.14: Agents are modelled in a 2D space. Cells migrate outwards in a circular fashion. In this simulation only contact inhibition of locomotion and self-propulsion are used. As time passes, cells spread out and the distance between cells increases. Contact inhibition is the key driver of the migration outwards.

In initial experiments to explore the effect of CIL alone, the adhesion term was set to zero, by setting $F_{adh} = 0$, meaning only repulsive forces and self-propulsion could drive cell locomotion. At early time points, the dominant term in the equations of motion is the repulsive contact inhibition (CIL) term. This is due to the geometry of the experimental set-up (agents are packed closely together as in the *in vitro* experiments) and the fact that the CIL term is proportional to distance between agents. Initially the agents are grouped tightly together, resulting in strong repulsive forces between agents, which due to the geometry of the initial condition results in a net outward force, as shown in Fig. 5.15. However, as they migrate out into space, the distance between agents increases, resulting in self-propulsion being the dominant form of motion, since there was no adhesive forces which would act to keep cells together and the mean distance between cells at the leading edge is greater than r_{sep} .

This explains why the amount of disorder increases at the leading edge, since although

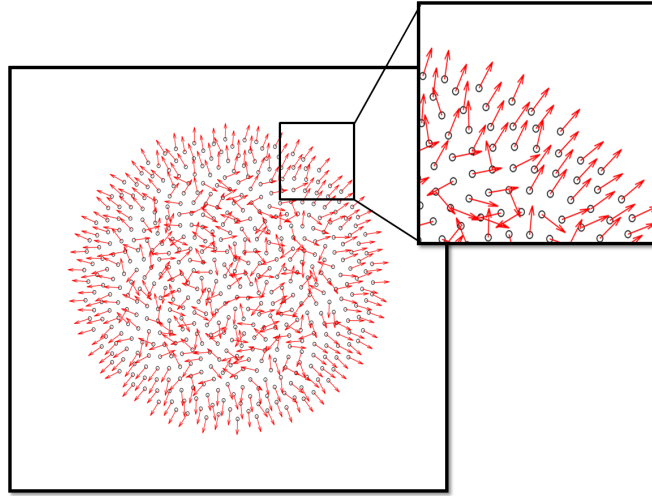


Figure 5.15: Contact inhibition results in a net outward force of cells at the leading edge. Red arrows show the direction of motion of cells (black circles). Arrows at the edge point outwards, showing how CIL results in directional motion when cells are close together.

CIL initially results in directional migration outward (due to the topography), the area occupied by cells grows proportionally to the square or step size, the area is greater at the edge, little or no adhesion between cell, means that the dominant term in the equations of motion is the self propulsion term. This is shown in Fig. 5.18A, where the ratio of external force to internal force (self-propulsion - SP) is shown. The magnitude of external forces decreases over time, becoming equal to SP force at $t = 75$, and continuing to decrease. The decrease in external forces is correlated with an increase in disorder (see Fig. 5.18B). The initial simulations resulted in a stabilised entropy which was lower than the *in vitro* experimental results, and a larger displacement and higher directionality. The variables to consider are 1) the radius of interaction for CIL, 2) the lack of adhesive forces, 3) the degree of randomness in self-propulsion term and 4) the ratio of the internal to external forces. Since the repulsive cues of CIL require cell-cell contact the radius of interaction cannot be reasonable changed. In the following sections I will explore how introducing adhesion and altering persistence

in motion can effect migration. Since cell migration was more ordered when the distance between neighbouring cells was small, I wanted to see whether increasing cell-cell adhesion would lead to an increase in order, by keeping cells close together and allowing the CIL induced order to be maintained or alternately decrease cell dispersal but increase disorder by disrupting the CIL force. I also investigated how persistence in self-propulsion of individual cells would impact the order of the migration. Finally, I looked at how adjusting the relative strength of internal vs. external forces on cell motion impacted on collective cell migration. These factors were introduced separately and the together and entropy and displacement metrics were used to quantify the effect on the model.

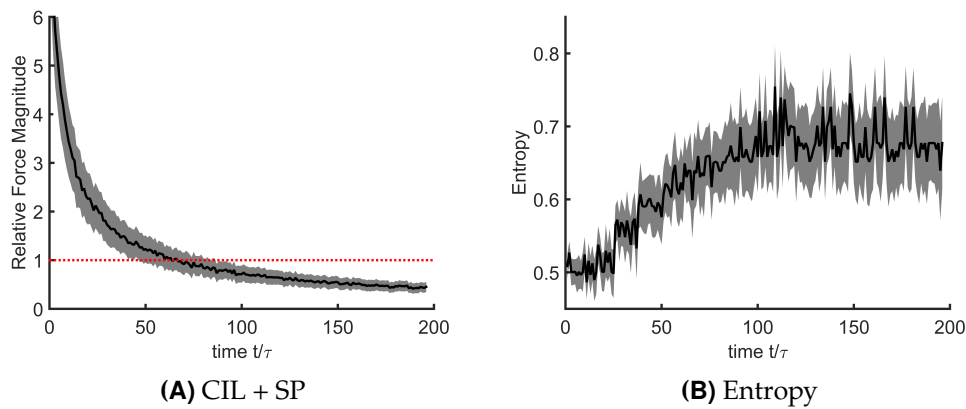


Figure 5.16: The relative force of external to internal motion forces for (A) CIL and self-propulsion only. When there is no adhesive force to keep cells together, cells move apart and the external force (CIL only) decreases overtime, resulting in self-propulsion becoming the dominant term in cell motion. This results in an increase in disorder (entropy metric) as shown in (B)

The effect of noise in simulations of Schwann cells migrating under CIL and self-propulsion

When the noise term in equation 5.7 is small in the self propulsion term, velocity correlations form over distances, however when it is larger, these correlations are lost. Low noise in self-propulsion increases cell displacement, and increase order in cell migration by allowing for velocity correlations to form. The importance of this is mediated by the ratio of internal (self-propulsion) to external forces.

If the external force is greater than the internal force at early time points, when cells are still close together contact inhibition between cells drives cell migration outwards (see Fig. 5.15) and noise in self-propulsion cell has little effect on order as shown in snapshots in Figs. 5.17A and 5.17B and quantified using local entropy in Fig. 5.17E. At early time-points, there is no significant difference (t-test, $n = 15$) between the local entropy of cell which have low or high persistence when the external forces dominate. However, at late time-points in the simulations, once the self-propulsion term is the dominant term, high persistence results in maintained order, while low persistence results in loss of order. This is clearly not an effect of cell-cell interactions however. When the internal force is greater than the external force, when the noise term is high, cell migration outward is disrupted (see fig. 5.17C), while when the noise term is low the migration outwards is maintained. This is heavily dependent on the initial internal direction of motion of the cells. These effects are quantified using the local entropy. In Fig. 5.17E, the aggregate local entropy for early and late time point is shown for each of the cases discussed above. Early and late time-points are defined as $0 : t_s$, and $t_s + 1 : 2 \times t_s$ respectively, where t_s is the time point in initial simulations where the relative force of external factors becomes equal in magnitude to internal motion.

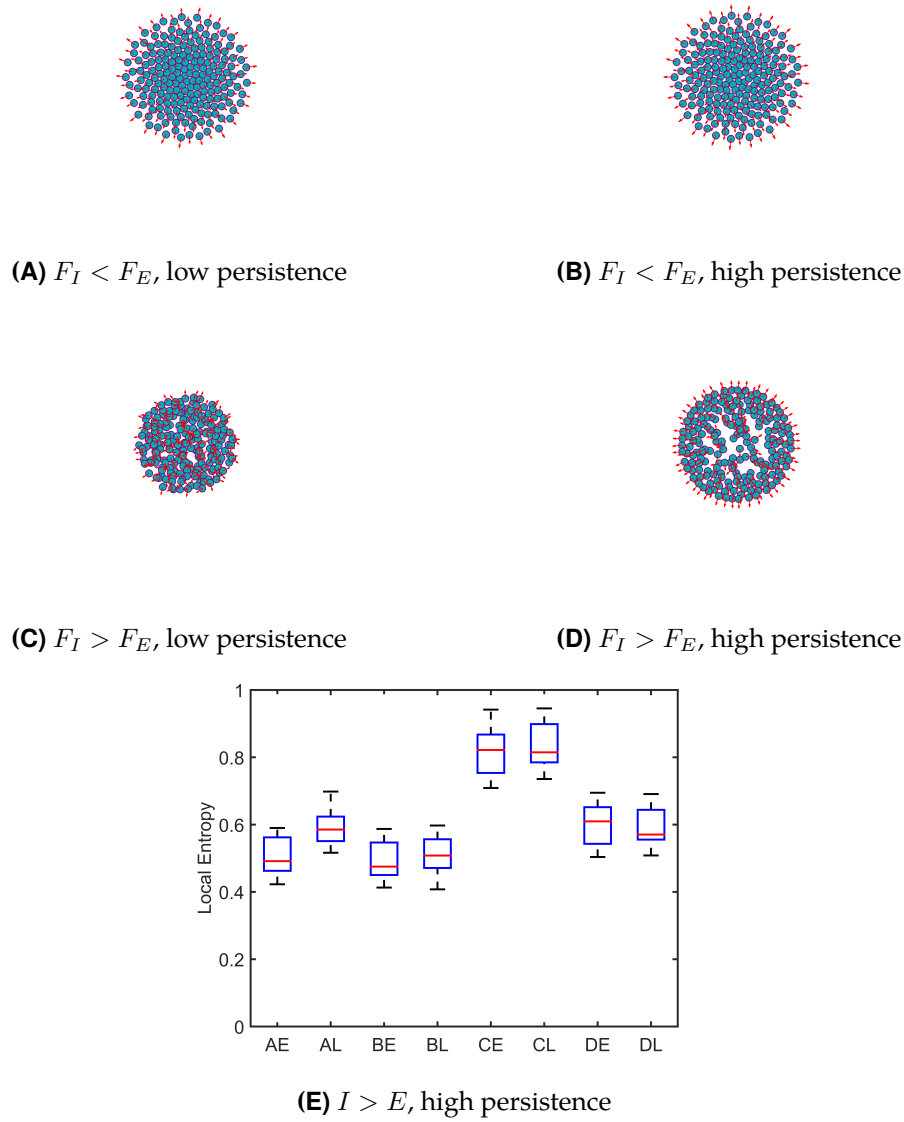


Figure 5.17: (A) - (D), When only CIL and self-propulsion are used to describe cell-locomotion, the effect of persistence in cell motion is (as expected) only clear when the internal forces (F_I) are greater than external forces (F_E). This is clear to see by the alignment of the . (E) Local entropy is used as a measure of local order, the first letter in data labels refers to the simulation conditions and corresponds to the Figs. (A)-(D), and the second letter in the data labels refers to early (E) or late (L) time-points.

Simulation of Sc Alone under CIL, adhesion and self-propulsion

To simulate some cell-cell adhesion, the adhesion force factor, F_{adh} is set to 0.5. This introduces a small adhesive force which should act to keep cells from spreading out. Visually, the superficial effect of increasing adhesion serve to reduce cell separation, as shown in Fig. 5.18. Cells are kept closer together and the radius of migration increases at a slower rate than when only CIL and self-propulsion are included. The dominant term in the motion equations are the external forces and seen in Fig. 5.19A, which arises because cells are maintained within the interaction radii by the adhesive forces and prevented from spreading out as under CIL alone, the external forces (CIL + Adhesion) are maintained overtime, while the self-propulsion term is of minimal importance. Increasing adhesion did not lead to an increase in order in cell migration, rather it served to increase the disorder between cells (see Fig.5.19A) and decrease the displacement (see Fig.5.21B). Although there is a net motion outwards, and cells are kept together, the local order is low.

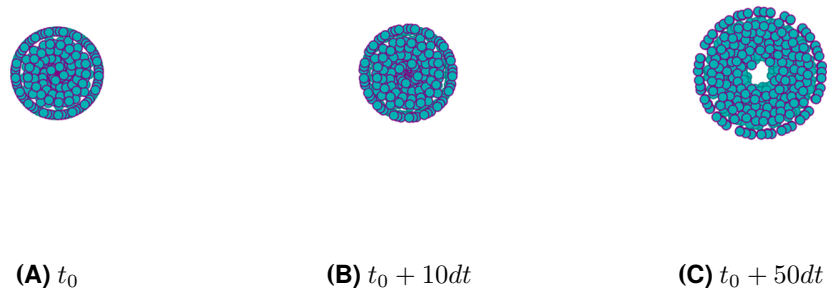


Figure 5.18: Snapshots overtime for simulations of Sc alone with adhesion, contact inhibition and self-propulsion. Agents stay closer together and the radius of migration increases at a slower rate than when only CIL and self-propulsion are included.

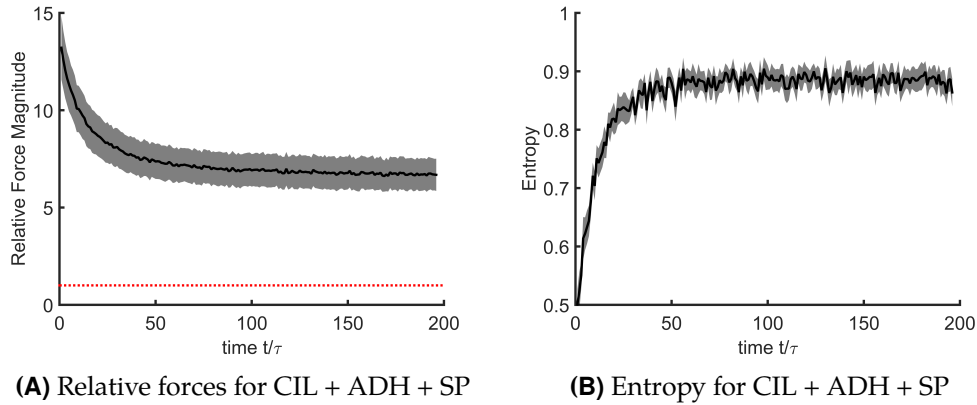


Figure 5.19: (A) The relative force of external to internal motion forces for adhesion (ADH), contact inhibition of locomotion (CIL) and self-propulsion (SP). The dominant term in the motion equations are the external forces (adhesion and CIL), which arises because cells are maintained within the interaction radii by the adhesive forces and prevented from spreading out as under CIL alone, thus, the external forces are maintained overtime, while the self-propulsion term (magnitude shown as the red dashed line) is of minimal importance. (B) Including the adhesion force does not increase order (seen by increase in entropy over time). The inclusion of the adhesion force results in more local interactions, which without guidance act to increase disorder rather than decrease it suggesting that adhesion alone is not sufficient for organisation.

Introducing heterogeneity into the adhesion levels of cells resulted in cell separation based on their adhesion level. As can be seen in Fig. 5.20, from an initially mixed population of cells, cells which have a high adhesion index (dark blue) cluster together and do not migrate outwards, whereas cells which have a low level of adhesion (teal) migrate outwards and do not form lasting cell-cell adhesions.

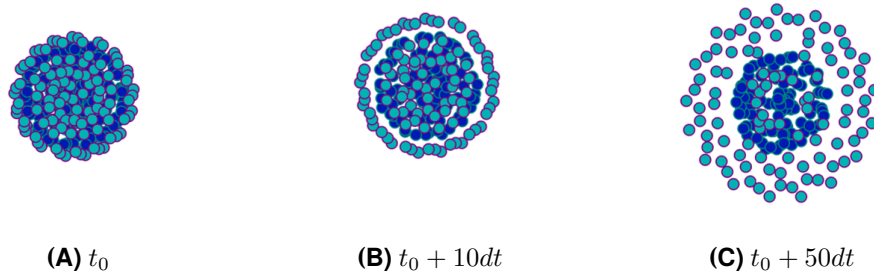


Figure 5.20: Agents are modelled in a 2D space. Cells migrate outwards in a circular fashion. In this simulation only contact inhibition of locomotion and self-propulsion are used.

Within the CCMAB model, CIL is a driving outward force, which is disrupted when high cell-cell adhesion is introduced or when self-propulsion in the dominant term and cell motion is random. From *in vitro* experiments, I have evidence that cell-cell adhesion is low and not maintained overtime. Furthermore, I showed that individual cell intrinsic motion has low persistence. The key question I wanted to use my model to explore was how cord like collective cell migration could arise. Introducing permutations into the model, within reasonable biological constraints could not recapitulate cord like migration. Using a mixed population of high and normal adhesive cells only resulted in population clustering based on cell adhesion. Therefore, although parametrizing against *in vitro* experiments of Schwann cells alone allowed me to recapitulate the form of migration observed, and I have shown that contact-inhibition induces migration but cannot explain cords and cannot maintain order in unconstrained environments.

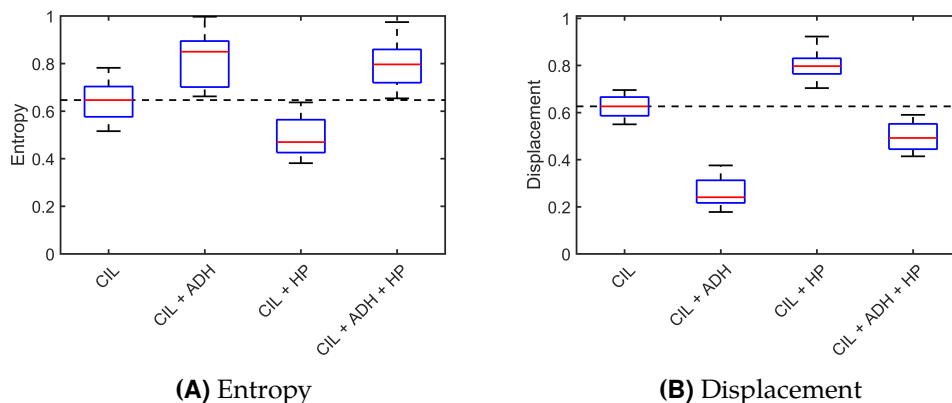


Figure 5.21: To understand the effect of contact inhibition (CIL), adhesion (ADH) and increased persistence on order *HP - high persistence) and cell migration (A) Entropy and (B) displacement metrics were used. Results are shown for 15 independent simulations. The dashed line shows the mean value of entropy (A) and displacement (B) for *in silico* simulations of CIL alone.

Sc + fbs Simulations

Having established the collective cell migration agent based model (CCMABM) model in the case of Schwann cells migrating into free space, I then used it to investigate what features are necessary and sufficient biologically for cord formation. Above I showed that under reasonable biological conditions, adjusting parameters of the model could not induce this kind of behaviour. In introducing fibroblasts into the model, two new features are introduced; (1) local repulsive guidance cues and (2) local cues which induce state change in Schwann cells. The term local is used to denote that these effects are only felt by Schwann cells once they come into contact (or in the simulation, within a certain distance) of the aforementioned fibroblasts. These constraints follow directly from experimental work, which is described in Chapter `chapt:scfbs`. In the following sections I show the impact of these features on the model, quantify the effects on migration and investigate their role in cord like collective cell migration. Since the model becomes significantly more complex, I use the cosine difference between scaled

global entropy, local entropy and directionality, to quantify migration behaviour. The cosine difference of two vectors, $\vec{a}$ and $\vec{b}$ is,

$$\theta = \cos^{-1} \frac{\vec{a} \cdot \vec{b}}{\|\vec{a}\| \|\vec{b}\|} \quad (5.19)$$

where $\vec{a}$ is the vectorised form of average results from simulations - $\vec{a} = [H_g, H_t, d/D]$, and $\vec{b}$ is the reference vector; the average results from experimental data. All data are bounded by limits $[0, 1]$. The value θ is a measure of the distance between the vectors, $\theta = 0$ for identical vectors and the larger θ the greater the discrepancy between the simulation and experimental data. The impact of individual measures can be looked at using the difference of squared values in each metric.

As shown in Chapter 3, fibroblasts change the behaviour of Schwann cells. Briefly, they become more adhesive to other Schwann cells, adopt an elongated morphology and exhibit a more directional individual migration. In my model, interactions with a fibroblasts results in agents undergoing a state change. I look at the impact of local state changes in Schwann cells on collective migration. When Schwann cells come within the interaction radius of a fibroblast, they change state, becoming more adhesive (higher f_{adh}), increase their adhesion radius and decrease their repulsive radius. As can be seen in Fig.5.22A, local state change leads to localised groups of high adhesive cells (dark blue) at the leading edge. This differs from the outcome observed in Fig.5.20, where cells were randomly assigned a high or low adhesion index. While this does lead to some distortion of the edge profile (particularly as time progresses as shown in Figs. 5.22B and 5.22C), persistent cords are not formed and cell migration outward is somewhat hindered, as reflected in the directionality (see Fig.5.25. Local increases in adhesion does not induce order, rather it leads to an increases disorder by confusing the simple repulsive signal from CIL, as can be seen in Fig.5.25, by using local entropy

as a readout of correlation in motion.

In addition to this change in phenotype, contact inhibition also occurs between Schwann cell and fibroblasts, thus, fibroblasts act as local repulsive cues. To understand if repulsive cues alone were sufficient to induce cord formation or organisation, Schwann cell agents which interacted fibroblast agents could now undergo CIL with fibroblasts but would undergo no adhesion state change. Although the fibroblasts initially act as guidance cues, separating cells out into narrow streams, lack of adhesion between cells means that they soon disperse, as seen in Fig. 5.22, and local interactions are no longer correlated because cells are not within interaction radii of their nearest neighbours. Thus, repulsive cues from fibroblasts were not sufficient alone to induce cord like migration in Schwann cells.

Finally I looked at the joint effect of combining adhesion, repulsive guidance from fibroblasts and persistence. Fibroblasts provide local guidance cues and play a dual role in a) changing the heterotypic state of the Schwann cells to become more adhesive and elongated in shape, and b) providing local guidance cues. Adhesion is necessary for cord formation, however the role of cell exclusion forces (volume exclusion or CIL) are also necessary for initial migration outwards and preventing back-flow of cells following interactions with fibroblasts (see Fig. 6.1). Individual features are not sufficient to replicate the behaviour observed *in vitro*, this was clear from observing the cell behaviour in the *in silico* model as well as comparing migration metrics (global entropy, local entropy and displacement) to experimental data. Optimising the combination of parameters which control adhesion and repulsion resulted in a simulation which represented the experimental data well (cord were formed and maintained - see Fig. 5.25 and Fig. 5.26), and migration metrics were in very good agreement with experimental

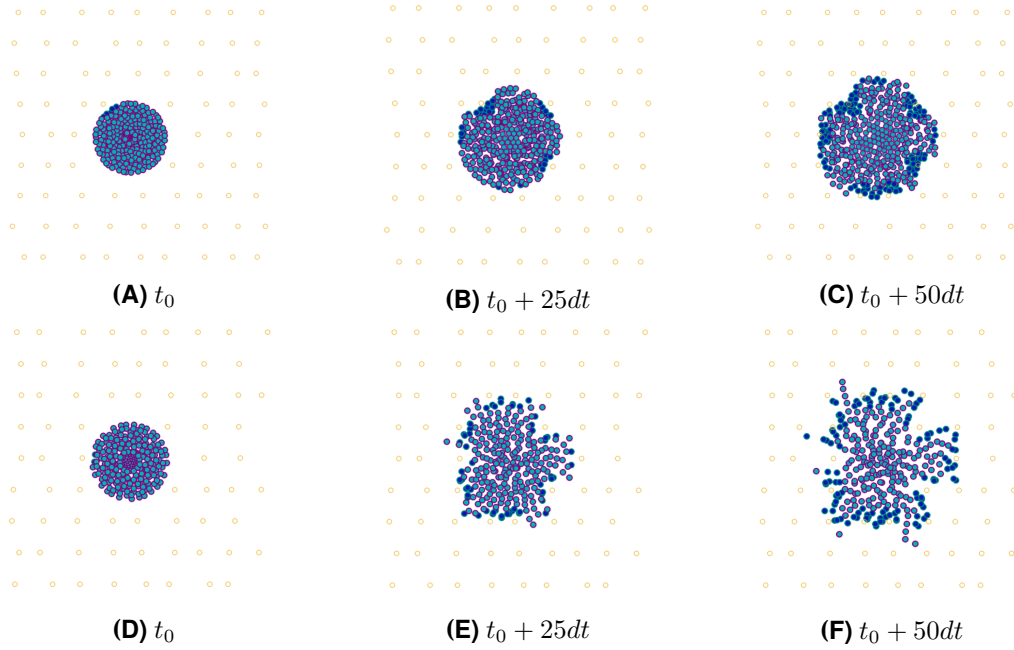


Figure 5.22: (A-C) In this simulation fibroblast interaction dependent adhesion, homotypic (Sc-Sc) contact inhibition of locomotion and self-propulsion are used. Cells which interact with fbs change state and become more adhesive, this leads to some distortion of the edge profile (particularly as time progresses (B-C)), however persistent cords are not formed. This suggests localised adhesion alone is not sufficient for organisation and cord formation. The cosine similarity = 0.4, and the largest difference in simulation to biological experimental data was due to local entropy. (D-F) In this simulation only homotypic (Sc-Sc) and heterotypic (Sc-fbs) contact inhibition of locomotion and self-propulsion are used. Schwann cells which interact with fbs (dark blue) do not change their adhesive state but do undergo repulsion, this leads to Schwann cells separating out (B), but as time progresses, without adhesion to keep Schwann cells together, Schwann cells disperse and no persistent cords are formed (C). This shows that localised repulsive cues alone are not sufficient for organisation and cord formation. The cosine similarity = 0.51, and the largest difference in simulation to biological experimental data was due to directionality. Due to cells spreading out and not being constrained, the distance travelled was greater than expected.

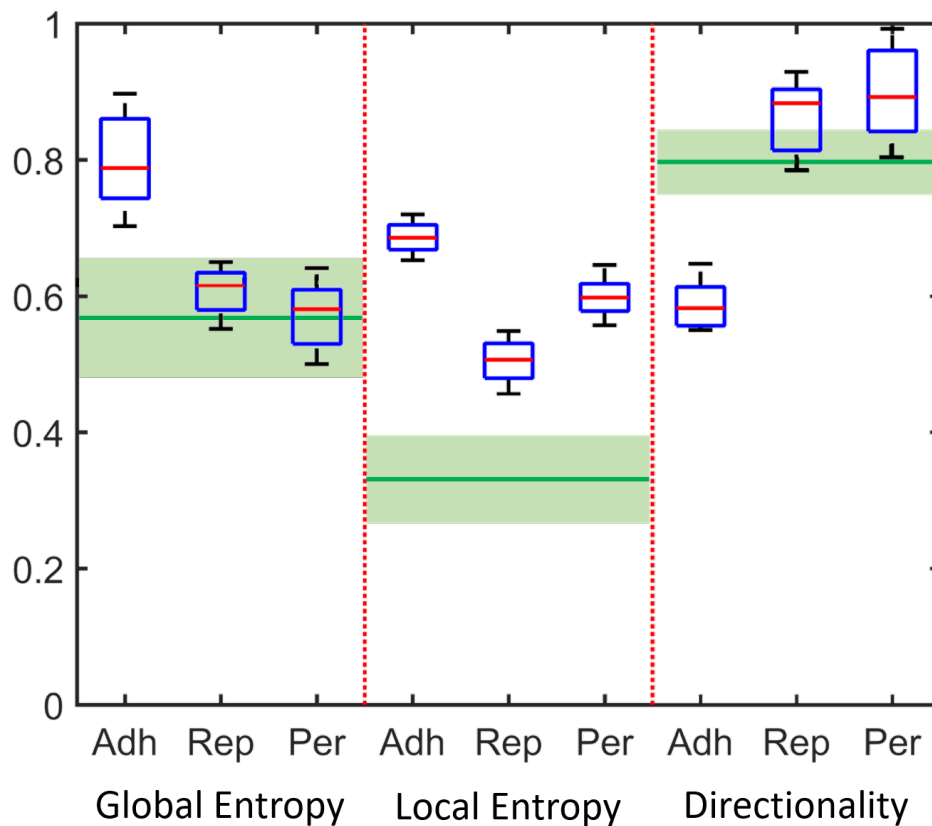


Figure 5.23: Global and local entropy and directionality are used to quantify migration behaviour in *in silico* simulations to study the effect of adhesion (Adh), repulsion (Rep) and persistence (Per) as cell behaviours on collective migration. Here global and local entropy are scaled by a factor $1/100$, 1 is maximum entropy (low order), 0 is minimum entropy (high order). Directionality is the displacement divided by the total length of the cell trajectory, values closer to 1 indicate a more directional route, while values closer to zero indicate a more meandering path. Adhesion - local interactions with fibroblasts induce state change to a high adhesive state in Schwann cells. Repulsion - A repulsive interaction occurs between Schwann cells and fibroblasts. Persistence - local interactions with fibroblasts results in a decrease in noise in internal motion. The solid green lines and shaded green areas represent the mean and standard deviation from measurements on collective cell migration in the *in vitro* migration assay ($n = 25$ experiments) for Schwann cells migrating with fibroblasts. In each metric (global entropy, local entropy and directionality) repulsion alone is , however . In the case of adhesion and persistence alone, a term for volume exclusion is included to prevent cells from becoming densely overlapping which is unrealistic.

data (see - local entropy Fig. 5.27C and Fig. 5.28)

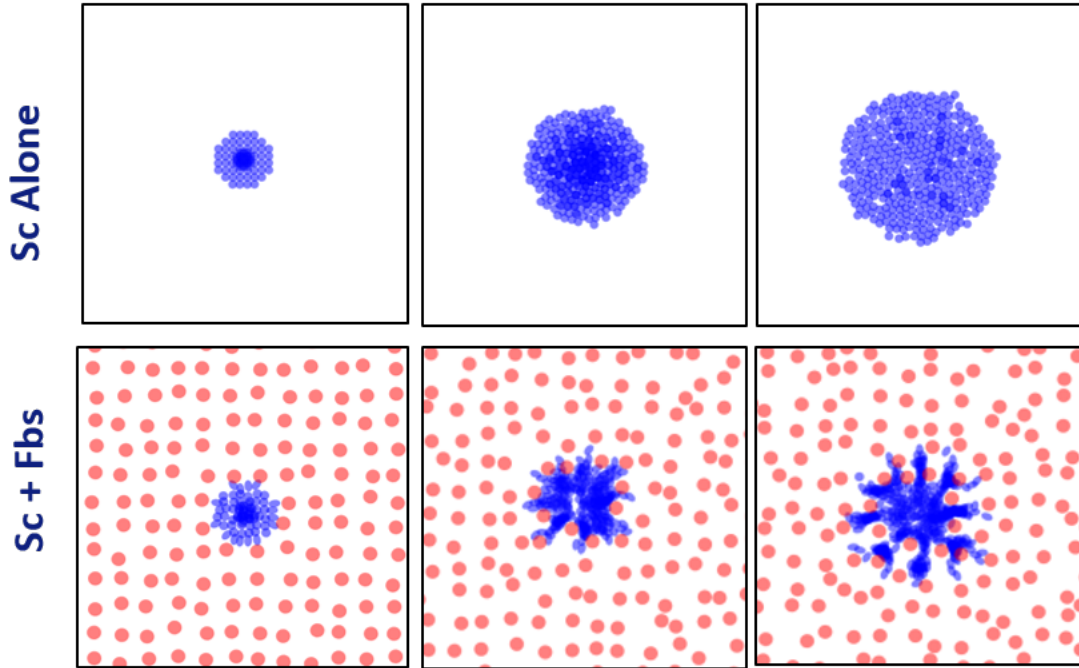


Figure 5.24: Snapshots of simulations (Sc in blue, fbs in red) with optimised parameters for Sc Alone (top), and Sc + fbs (bottom) for times (left to right): $t = t_0, t_{0+25}, t_{0+50}$. When Sc are alone, cells migrate outwards primarily due to CIL, however self propulsion and the associated noise also contributes to motion. Sc + fbs, when fibroblasts act to (1) increase adhesion between cells, (2) increase persistence in individual cell motion, (3) act as local guidance cues.

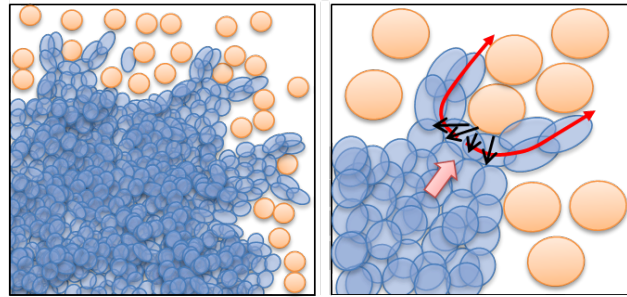


Figure 5.25: Schematic of interaction of Schwann cells (blue circles) and fibroblasts (orange circles). Repulsive cues on Schwann cells from Schwann cells (pink arrow) and fibroblasts (black arrows) and the resulting direction of motion of Schwann cells (red arrows).

Effects of EphrinB2 on Schwann cells

In the model I found that a combination of adhesion, low noise and repulsion both from local Schwann cells and fibroblasts is necessary for directional cord like collective migration. These behavioural features are induced by contact dependent signalling with fibroblasts. My model predicted that when cell-cell adhesion is lost, Schwann cells would lose the ability to migrate in a persistent and directional manner. This prediction is confirmed using $\text{ephrinB2}^{-/-}$ fibroblasts which results in loss of direction and cord formation in collective migration. EphrinB2 plays a key role in signalling between Schwann cells and fibroblasts. EphrinB2/EphB2 signalling results in re-localisation of N-cadherin.

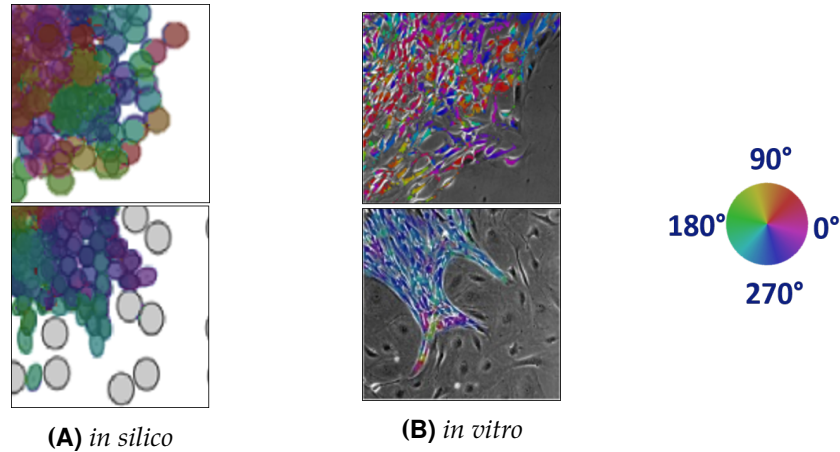


Figure 5.26: Comparing collective cell migration in *in silico* to *in vitro*. The same measurement (optic flow) and quantification methods can be applied to *in silico* simulations of cell migration and to *in vitro* cell migration data. (A) agent direction of motion (at a time-point) is visualised by overlaying the directional component of the optic flow field onto the *in silico* simulation. (B) cell motion direction is visualised by overlaying the directional component of the optic flow field onto the phase contrast image. Direction of motion is indicated by colour, the colour-map of direction is indicated in the colour-wheel. Both *in silico* (optimised model) and *in vitro* the direction of cell motion has little order when Schwann cells are alone (A,B top) indicated by neighbouring cells going in different directions, whereas when Schwann cells migrate with fibroblasts (A,B bottom) the range of colours is much more narrow, and neighbouring cells have similar colours. This is quantified using global and local entropy (see Figs.5.27C and 5.28B).

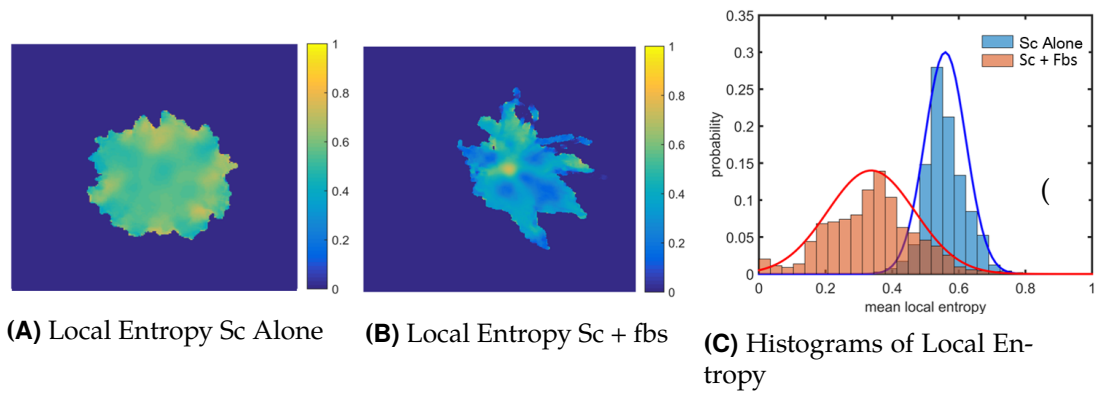


Figure 5.27: Local Entropy maps of simulations for (A) Sc Alone and (B) Sc + fbs for time, $t = t_0 + 70dt$. When there are no fibroblasts (A) no persistent cords forms, and the mean projected local entropy is in the region ≥ 0.6 . The cord like structures are evident when fibroblasts are included in the simulation (B), and the mean local entropy overtime appears lower, typically much less than 0.8. Fibroblasts are not shown as the local entropy is calculate from optic flow which is calculated from images with fbs masked out. (C) The distribution of the local entropy over all time is shown for Sc Alone (blue) and Sc + fbs (Orange). When Schwann cells are alone (blue plot), the local entropy is peaked around 0.6, and has a small spread indicating the local order is quite constantly low). When fibroblasts are present (orange plot), the mean local entropy decreases to 0.38, but the spread increases indicating that there are larger changes in local order, but overall order is high.

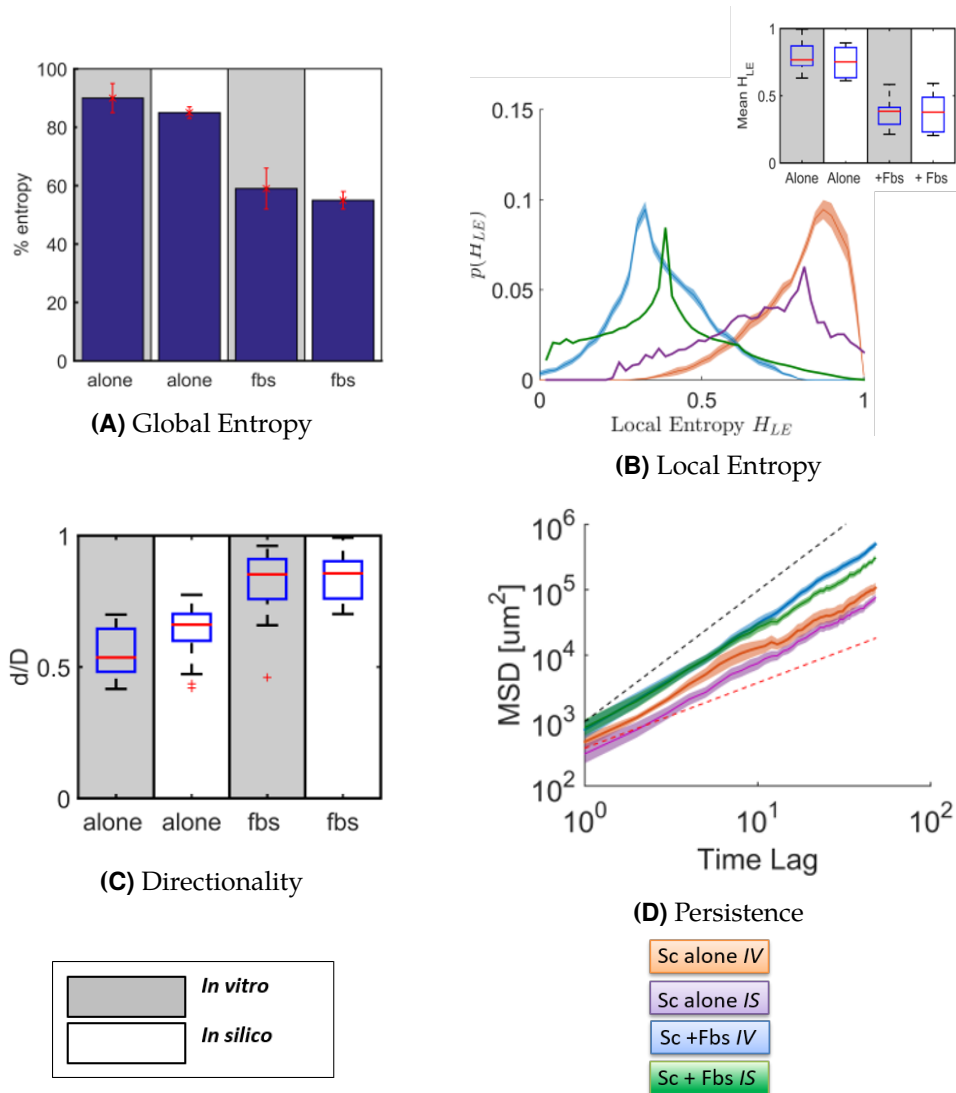


Figure 5.28: By optimising the free parameters in the *in silico* model the simulation not only gave similar superficially results (behaviour observed by eye - see Fig.5.24 for snapshots) to the collective cell migration seen in the *in vitro* experiments, but also gave similar results in quantitative metrics. (A) Global entropy, (B) distributions of local entropy, (C) directionality of cell motion, (D) mean square displacement are used to measure cell migration. By using these methods we know that the superficial similarity is caused by cell interactions which results in the same type of quantitative readouts with respect to order (global/local entropy) and mode of migration (displacement/MSD). Results from *in vitro* experiments are shown with a grey background, results from *in silico* simulations are shown with a white background.

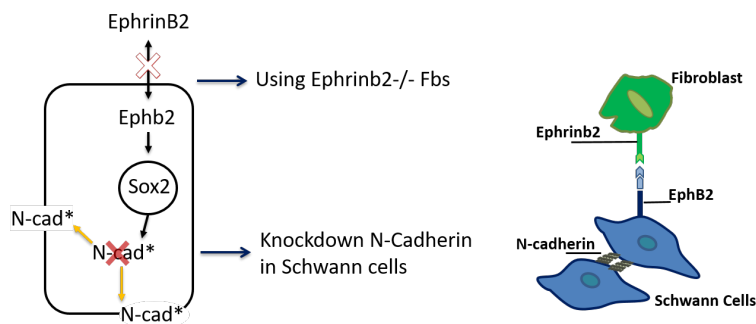


Figure 5.29: N-cadherin is re-localised to cell-cell contacts following ephrinB2/ephB2 signalling between Schwann cells and fibroblasts. Using ephrinB2^{-/-} fibroblasts in the migration assay is used to test the role of Ephrin in successful cord formation.

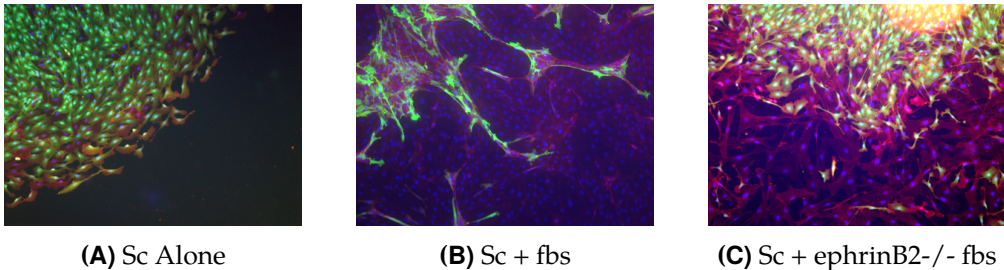


Figure 5.30: Comparing migration of Schwann cells alone (A), Schwann cells with fibroblasts (B) and Schwann cells with ephrinB2^{-/-} fbs (C). The typically cord like formations are clearly lost when ephrinB2^{-/-} fibroblasts are used.

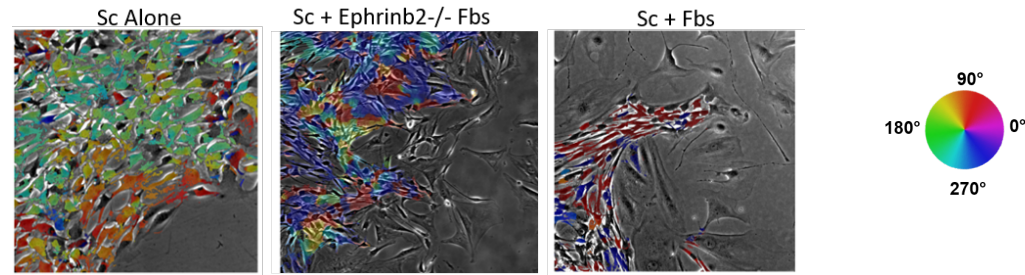


Figure 5.31: Using optic flow in *in vitro* experiments to determine the role of ephrinB2 in collective cell migration. Directional optic flow field shows the lack of local order in Schwann cells migrating with ephrinB2^{-/-} fibroblasts (middle), it appears more similar to the directional field of Schwann cells migrating alone (left) than Schwann cells migrating with normal fibroblast (right).

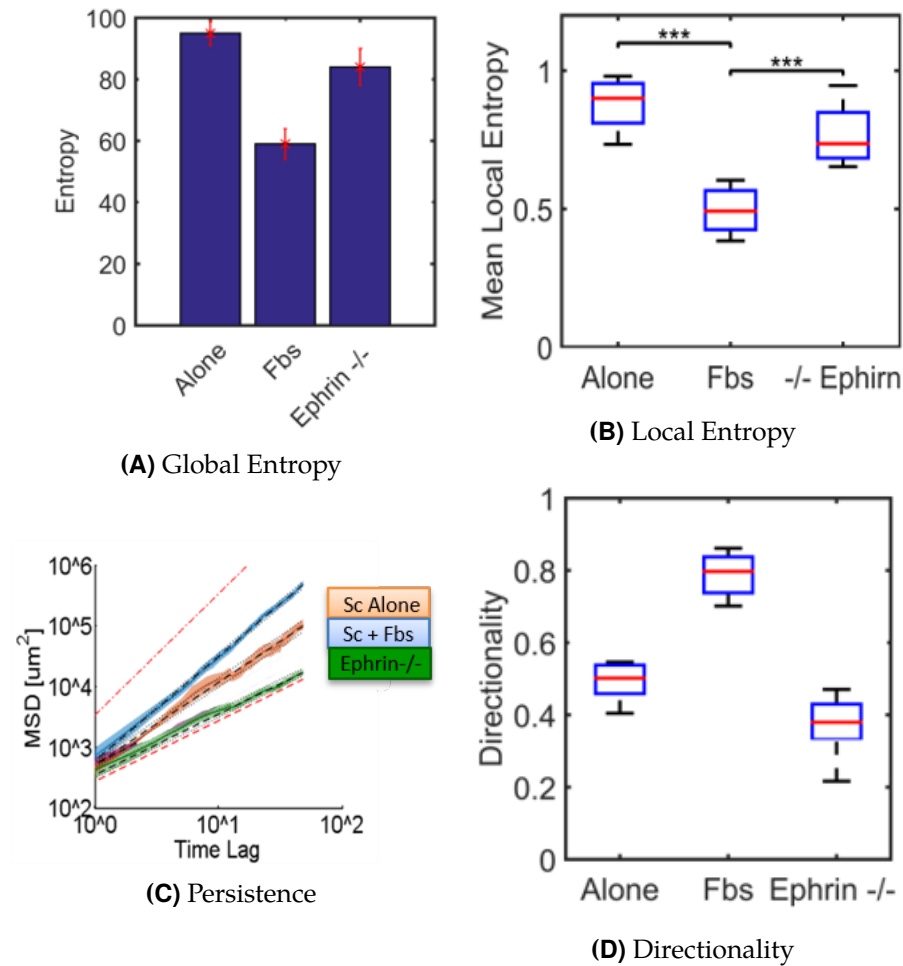


Figure 5.32: To compare *in silico* and *in vitro* experiments entropy and measures of persistence are used. Different measures capture different aspects of cell migratory behaviour. Entropy can capture order (locally and globally) while measures of persistence and directionality capture how cells migrate overtime. (A-B) Sc with ephrinB2^{-/-} fibroblasts exhibit higher disorder (high local and global entropy), a behaviour which is more consistent with Sc Alone migration than Sc with normal fbs. The MSD (C) and directionality (D) also decreases, and are lower than when Sc are alone. This suggests that some interaction with fibroblasts (volume exclusion/non ephrinB2 mediated signalling) is occurring.

6

Discussion

6.1 Overview

Collective cell migration is the coherent movement of multiple cells connected via cell-cell adhesion. At the cell level, it is well known that cell-cell adhesion, polarisation and contact inhibition of locomotion can direct collective cell migration. Given the complexity of collective cell migration, I used an integrative biological and computational approach, and find evidence that contact dependent signalling between Schwann cells and fibroblasts via ephrinB2/ephB2 plays a key role in regulating the interplay between these features.

In my research, I have been trying to understand how the interaction between Schwann cells and fibroblasts leads to a change occurring in the mode of Schwann cell migration from single-cell migration to coherent collective migration. From previous work in this system (Parrinello 2010), it is known that signalling between fibroblasts and Schwann cells is necessary for successful repair. I use this as a model system to investigate how directional collective migration can emerge from heterotypic cell-cell interactions between Schwann cells and fibroblasts. I built an agent-based model, in which the behaviours of agents are based on features extracted from *in vitro* cell and molecular biology experiments, and parameterized the model using quantitative metrics of collective cell migration derived from *in vitro* live cell imaging experiments. I then used the model to investigate how cord like collective migration can arise from local changes in the behaviour of Schwann cells mediated by Schwann cell-fibroblast interactions.

In *in vitro* migration assays, Schwann cells migrate as a loose sheet. Following direct contact with fibroblasts, they instead adopt a more motile state, becoming polarised in the direction of motion, increasing adhesion between neighbouring cells and migrating in a directional, cord-like fashion. Using optic flow, I measured the whole field motion and developed a set of quantitative measures derived from statistical physics (e.g. global and local entropy) to characterise collective migration. These parameters enable us to quantify collective migration and understand both the short and long range dynamics.

Key hallmarks of collective migration are that groups of cells migrate in the same direction at similar speeds and coordinate their response to the environment. Collective cell migration is an essential phenomenon in many biological events, from the start of life during development, and later in wound healing, repair and pathological events. It is a complex phenomenon, as it involves a large number of cells which are interacting not

only with the environment but with each other. For cells to coordinate motion and stay together, local cell-cell communication is essential. Thus, both mechanisms of single cell migration and direct cell-cell signalling play important roles in CCM. I wanted to understand how signalling and the resulting phenotypic changed in Schwann cells could lead to a change in the mode of collective cell migration. In designing and using an *in vitro* migration assay wherein the presence of fibroblasts resulted in a change in the mode of migration and the formation of cord like structures. I showed that fibroblasts impact cell-cell adhesion in Schwann cells, their cell morphology and motility. Following interaction with fibroblasts, adhesion between Schwann cells increases, they adopt an elongated morphology, and their motility becomes more directional.

To do this, I have carried out both biological *in vitro* and live cell imaging experiments and computational simulations. I used methods from image processing and computer vision to extract quantitative data from experiments. I then used methods inspired from statistical physics to find quantitative measures of cell behaviour, which allowed me to gain a deeper understanding of complex events. My computational model is an agent based model, and the properties and behaviours of the agents are based on data from biological experiments and are parametrised against quantitative measures of behaviour which characterise the interactions of Schwann cells and fibroblasts. Using my model, I investigated how Schwann cell-fibroblast interactions can result in directional collective migration. I wanted to understand how individual interactions in a discrete model give rise to emergent population-scale patterns. Particularly, how a local change of state (change in behaviour) can result in cord like collective migration.

6.2 Findings and Implications

I have presented a method for extracting complementary quantitative measures of order in collective migration. These take into account alignment, cohesion and persistence of collective migration cell at local and global scales. Using these methods, I studied the effect of contact inhibition, adhesion and local guidance cues on cell migration by using an agent based model.

I showed that fibroblasts impact the state of Schwann cells, changing their shape, increasing homotypic adhesion, decreasing contact inhibition of locomotion and increasing motility. I also showed signalling via ephrinB2/ephrB2 results in re-localisation of the adhesion protein N-cadherin, and change from rounded to elongated morphology. To understand how these phenotypic changes result in directional collective migration I built an agent based model with rules based on phenotypic data from our experiments. Calibrating the model parameters against *in vitro* migration assay data using our quantitative methods, I explored the interplay between adhesion, contact inhibition of locomotion and polarisation to determine the importance of these factors in collective migration.

I found that a combination of adhesion, polarisation and separation is necessary for directional cord like collective migration. I showed that these features are induced by contact dependent signalling with fibroblasts. My model predicts that when cell-cell adhesion is lost, Schwann cells lose the ability to migrate in a persistent and directional manner. This prediction is confirmed using ephrinB2^{-/-} fibroblasts which result in loss of direction and cord formation in collective migration. From the analysis of *in vitro* live cell imaging experiments I found that when Schwann cells migrate alone, there are clear features of CIL, at the edge especially. Schwann cells move as a sheet, which becomes loose at the edge as the amount of free space increases. Adhesions between

cells are transient, we see this as 1) clusters do not form, 2) they are in 2D random or uniform in space, 3) when migrating as sheets where there is space they spread out. When fibroblasts are present in the migration assay, cell-cell adhesions are no longer transient, we see this as 1) clusters form, 2) although cells are still motile, they do not break up, 3) when they migrate in the migration assay, they form cords. Schwann cells migrate around fibroblasts, implying fluidity in cell-cell adhesion. Re-localisation of N-cadherin (to cell-cell contacts) rather than an increase in N-cadherin is important in the increase in cell-cell adhesion, and by quantifying the increase in the level of N-cadherin at the cell junctions (50% increase), suggests that adherens junctions are now more stable/stronger and so can overcome the CIL repulsive force

Simulations of migrating cells exhibit a good agreement with experimental results. Although cells at the edge of the spreading colony are highly motile, a high-tension non-motile state emerges in the tissue interior as cells divide that is driven by a feedback between CIL, cell density, and cell-cell adhesion. The cell-cell interaction forces vary as a function of the distance between individual particles, which means there is a linear relationship between cell density and inter-cellular forces. Thus, the densely packed cells in the centre of the group experience high repulsive forces, while cells at the edge exert only weaker forces on each other. When cells have a significant adhesive potential (from interaction with fibroblasts), cells at the edge maintain a connection to their neighbours, reducing cell separation.

The nature of this form of migration implies that high fluidity and dynamic adhesion is necessary, as although cells migrate in sustained cords, they also migrate around fibroblasts and can exhibit dynamic behaviour when repulsion from fibroblasts causes a direction change. For cord like migration to occur, a combination of adhesion, repulsion and local guidance was essential. Whether this could be enhanced by the addition of more complexity in the model (such as phenotypic features of leader cells)

would need to be addressed in future work. It is clear that collective motion is, rather, a result of the complex interplay between intra- and intercellular forces.

6.3 Limitations

There are two main limitations in my research, the first is that the experimental data were collected *in vitro* which has two implications on the environment in which cells migrate: (1) the migration is in a 2D environment, (2) the environment is lacking in other key cells and structures such as phagocytes, the ECM and the vasculature. Single cell and collective cell migration occur in complex environments, where they encounter many different cell types and move in a 3D extracellular matrix. Both of these factors affect the way in which the cells move, and the state the cell is in. Specifically, in peripheral nerve regeneration, we know that in addition to fibroblasts, there are macrophages, phagocytes, blood vessels, and later, regrowing axons.

In cell migration, cells can move in a 2D plane (for example endothelial cell surfaces of the circulatory system) as well as a 3D conditions (interstitial extracellular matrix of tissues), however the majority of current research has focussed on migration in 1D (along a line) or 2D (in a plane). Cell migration on two-dimensional (2D) surfaces is well characterized; cells extend lamellipodia at the leading edge, adhere to the substrate and then retract the trailing edge. However, the mechanisms underlying 3D migration *in vivo* are not well understood; It has been shown that cells can switch between lamellipodium-based and lobopodium-based migration in 3D matrices, and that this depends on both the degree of polarization of intracellular signalling and the physical properties of the extracellular matrix. Thus modelling 3D cell migration is significantly more than simply increasing the dimension space. To recapitulate 3D environments, collagen gel can be used, however, it is a significant challenge to image

cell migration of large numbers of cells in this setting. The techniques for *in vivo* live cell imaging are developing, however, it is still a significant challenge to record data which can be quantitatively analysed to enable validation and parametrization of such models, which are intrinsically more complex. Models should be used in conjunction with experimental data, therefore, although I think 3D modelling and either live cell imaging of either 3D *in vitro* experiments or *in vivo* is the ideal direction, it was not feasible for this work.

Since previous work had shown the interesting interaction of fibroblasts and Schwann cells, there was a strong motivation to investigate this further. It is evident that fibroblasts have an important effect on Schwann cell phenotype, and this effects their mode of migration. However, the effect of these other cells which are known to be present at the wound site would be an interesting area for further research.

Modelling in Biology

Computational and mathematical modelling can be a powerful tool in understanding biologically observed phenomena which cannot be understood solely via verbal or logical reasoning. When working with a model it is important to clarify why a model is being used, and what it can do that conventional biological experiments cannot. In designing and implementing a model of a biological system, there are a number of challenges which make it different from a model of a physical system, primarily these are the complexity of biological systems which can lead to difficulty in parameterising the model.

A model is defined as “a simplified or idealised description, representation or conception of a particular system, situation, or process, often in mathematical terms, that is put forward as a basis for theoretical or empirical understanding, or for calculations, predictions, etc.” Models should be as simple as possible, yet as complex as necessary

to address a given question of interest. Simplification allows us to comprehend the essential features of a complex process without being overwhelmed by unnecessary details. The key challenge is to correctly identify what to and/or simulated. As the eminent statistician George Box wrote, “essentially all models are wrong, but some of them are useful.” Box also draws attention to an issue of importance in modelling; “since all models are wrong the scientist must be alert to what is importantly wrong. It is inappropriate to be concerned about mice when there are tigers abroad.”

In my work, I use an agent based model, wherein cells are represented as discrete entities which interact according to their state. The advantage of this type of model is that it is easy to see it as an analogy of the real system and it is modular so can easily incorporate individual properties of cells. The disadvantage of this type of model is that the lack of rigorous mathematical theory (which would be found in a continuous model of differential equations) makes it difficult to determine how the effect of the assumptions made impact the system behaviour. It is also more challenging to check how robust behaviours are to changes in parameter values (i.e. whereas in a mathematical equation based model this can be more easily checked via an analytic solution). Instead, numerous simulations are required to be run. In practical terms, and analytical model -deriving a continuum model- wherein cells are represented as densities, would be advantageous for rigorous mapping of global parameters and the associated analysis and description of model behaviour. However, there is a significant challenge in encoding cell-level properties in these models.

An important aspect of my approach in this work is that cell-based rules are implemented in the model from biological observations and measurements. This gives the model some biological meaning and allows us to ask questions about the system, rather than just ask if we can find a generic emergent behaviour from playing around with parameters. Computations models, once parametrised and tested, have the potential

benefits of helping to design further experiments or even used for *in silico* experiments, which would be impossible or extremely challenging *in vitro*. I use my model to explore some scenarios which would be impossible or extremely challenging *in vitro*, to try to understand the requirements of the system. Simulations make useful, experimentally verifiable predictions, decreasing the adhesion of the agents causes a transition from cohesion to random motion, increasing the noise in the system can disrupt directed motion. These simulations are best used in conjunction with biological experiments as a successful model shows sufficiency of mechanism, but not a necessity. Consequently, we are most likely to identify new mechanisms when our simulation results differ from experimental results, implying that we are missing a key component in our model.

In describing and implementing the model I encountered the following challenges which are worth discussing. (1) Implementing behaviour: The difference between CIL and volume exclusion. (2) Combining cell behaviours.

Implementing Behaviour

Contact inhibition of locomotion (CIL) occurs when a cell stops migrating in a particular direction when it comes into contact with another cell. This was one of the features observed in our *in vitro* experiments. Schwann cells exhibit contact inhibition of locomotion when interacting with one another, however, this is greatly decreased when they are in the presence of fibroblasts, where either the adhesive interaction between cells seems to overcome any CIL or some downstream effector of ephB2/ephrinB2 signalling inhibits homotypic CIL.

Repulsive signalling plays a prominent role in regulating cell–cell interactions and is fundamental to multiple migration processes. The opposite action, cell-cell adhesion is a hallmark of collective migration. A proper balance between repulsion from and

adhesion to other cells is important for collective migration. When cells come into contact with each other, cadherins at the site of contact form trans-bonds between the cells. Once engaged in trans-bonds, cadherins can regulate the formation of cell–cell contacts in three distinct ways: by reducing the local intercellular tension 1) directly through adhesion tension and 2) indirectly through signalling to the cytoskeleton, and 3) by establishing the mechanical coupling of contacting cells.

Although the model I use focusses on cell-level behaviour, rather than mechanistic processes as described above, having a better understanding of the processes can lead to a clearer understanding of how to implement different behaviours within the model.

Combining Cues

In directed cell migration, cell motion is influenced by multiple migratory cues. These cues may include simultaneously occurring attractive soluble growth factor gradients and repulsive effects arising from cell–cell contact (CIL), adhesion between cells, and interaction with the environment. How cells reconcile or integrate potentially conflicting cues is not clear and it is a deeply interesting question.

The interaction forces (repulsive and adhesive) result from independent interactions and need to be combined using some method. However, there are a number of ways in which this could be imagined to happen, and different rules for combining behaviours can lead to very diverse outcomes.

In terms of cells, any one cell may interact with multiple other cells determine at any given time. Each velocity is associated with a "strength", and these are combined to determine a single final direction of motion, which the cell will attempt to move in. Regarding finding a parallel in biology, this strength could be thought of as being proportional to the concentration of an essential component in these interactions. For

example, in adhesion, the interaction strength might be a function of the amount of available N-cadherin or a transport molecule for N-cadherin.

The easiest way to combine acceleration requests is to average them, because of the included "strength" factors this is actually a weighted average. The relative strength of one behaviour to another can be defined this way, but it is a precarious interrelationship that is difficult to adjust. An alternative possibility could be that a cell can only perform one action at any given time and for a given state (phenotype) one action take is allocated. Given the complexity of a cell this seems unlikely, however, a case can be made for a cell prioritising one action over another. In this case, interaction forces might be combined in a prioritised manner, where the cell state defines the priority ordering of all component behaviours, i.e CIL is more important than adhesion. Although explicit decision to do this seems unlikely in a cell, an equivalent mechanism dictated by finite amounts of some essential protein or within the cell could be conceived of.

In my model, the strength of cell-cell interaction dependent cues are a linear function of distance, and cues are summed together to give an overall effect. At the scale (i.e. cell behaviour rather than cell signalling networks) these interactions could be modelled as a non-linear function (i.e. using the Morse potential or Lennard-Jones potential), or each behaviour could be modelled by different functions. It is not clear which interaction model to use. I used the piece-wise linear model as it is simple to implement and understand, however, a more complex model might be more representative of biological interactions.

6.4 Interesting areas of future research

A major difference between collective cell migration and single cell motility is the importance of continued communication between cells. In particular, the regulation of cell–cell adhesion differs substantially. Whereas cells migrating alone have little cell–cell adhesion to facilitate their movement, collectively migrating cells by definition maintain cell–cell attachments. An open question is what mechanisms maintain relatively stable adhesion between the cells of the cluster so that they stay together but allow transient adhesion on the outside surfaces of the same cells, so that they gain traction without getting stuck.

Molecular biology experiments show that the amount of N-cadherin in Schwann cells which have undergone ephrin/eph signalling either via interaction with fibroblasts or from being directly plated onto ephrin coating does not change. This evidence suggests that the spatial location of N-cadherin rather than the quantity is responsible for the increased adhesion between cells. However, it is unclear whether the increase in adhesion is due to an increase in the number of adhesion junctions formed or stabilisation of adhesions formed. The biological sequence of events leading to the formation of mature adhesion junctions has been studied in depth (Harris and Tepass 2010). Adherens junctions are assembled by the formation of cadherin–catenin clusters which form following contact between the lamellipodia of neighbouring cells. Subsequently, F-actin polymerisation is stimulated near to the clusters, allowing the junction to expand through extended contact of the lamellipodia (Yamada et al. 2005).

Following formation, the dendritic lamellipodial actin network is remodelled into a peripheral actin belt and network rearrangement by myosin contraction and, potentially, α -catenin bundling (Drees et al. 2005; Harris and Tepass 2010). Later, the actin belt becomes increasingly contractile, a process regulated by the crosstalk between

the small GTPases Rac1 and RhoA. Regulation of cadherin-mediated cell-cell adhesion is determined by distinct protein interactions of the cadherin extracellular and cytoplasmic domains. Binding between cadherin extracellular domains is relatively weak, but cell-cell adhesion may be strengthened by lateral clustering of cadherins mediated by protein linkages between the cadherin cytoplasmic domain and the actin cytoskeleton.

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