ABSTRACT

JOHNSON, HEATH ELLIS. Cytoskeletal and Signaling Dynamics Underlying Directional Persistence of Cell Migration. (Under the direction of Professor Jason M. Haugh).

Control of cell migration and directional persistence underlie many important physiological processes, from wound healing to metastasis. Mesenchymal cells, such as skin fibroblasts, migrate in a manner that is characterized by multiple competing protrusions, weak polarization, and strong adhesive forces. External cues such as chemical gradients and substrate stiffness work in concert with internal signaling events to direct cell movement. Efficient reorganization of the actin cytoskeleton in response to those signals is critical; however, the manner in which cells interpret internal and external cues and translate them to bias migration is poorly understood.

The lipid kinase phosphoinositide 3-kinase (PI3K) has well known roles in cell proliferation and survival and has been broadly implicated in cell migration. However, its specific role in the context of mesenchymal migration has yet to be elucidated. In this work, we describe a PI3K-dependent mechanism by which mesenchymal cells achieve large-scale reorientation of migration. We found that they do so by forming branched lamellipodia; if extension of the lamellipodia propagates to the fullest extent, the cell performs a ninety-degree turn. Inhibition of PI3K did not significantly reduce the instantaneous speed of migration or the frequency of branch formation, rather it abrogated the stability and propagation of the branched state. Thus, a PI3K-inhibited cell typically exhibits an elongated morphology and migration biased along its long axis. Dynamic recruitment of PI3K activity apparently responds to protrusion, and accordingly we found that it can be induced via actin
polymerization spurred by photo-activation of the small GTPase, Rac. Conversely, inhibition of actin polymerization prevents protrusion and dynamic relocalization of PI3K. In cell migration directed by an external gradient (PDGF chemotaxis), the branch that is exposed to the highest concentration of chemoattractant is favored, resulting in alignment of the cell up-gradient.

Having established a critical role for PI3K and lamellipodial branching in mesenchymal migration, we sought to discover the mechanisms by which new branches were formed and how PI3K activity originated in these structures. Analysis of high-resolution migration movies revealed that finger-like actin bundles called filopodia often precede and direct the formation of new lamellipodia. Using quantitative image analysis protocols that we developed, we showed that nearly all lamellipodia are biased by filopodia or submembranous actin bundles. To test this further, we depleted as well as overexpressed the actin-bundling protein, fascin-1, in different populations of cells and quantified the number of morphological extensions. As expected, the number of cellular extensions varied according to the fascin levels. Based on the finding that actin bundles template lamellipodial protrusion, we hypothesized that they also prime the recruitment of PI3K signaling. Using ratiometric imaging, we found that PI3K is indeed enriched within filopodia. By imaging focal adhesions and perturbing cells with pharmacological inhibitors, we found that adhesions serve as hubs for PI3K signaling primarily through the recruitment of focal adhesion kinase (FAK). This adhesion-based pathway fosters actin polymerization, dilation of lamellipodia, and the formation of new adhesions, thus amplifying signaling; under some conditions, this process manifests as periodic protrusion waves. Depletion of fascin ablated
direction migration in response to a fibronectin gradient (haptotaxis) but not PDGF chemotaxis. As fibroblast filopodia contain primed integrins at their tips which bind specifically to fibronectin, this suggests a possible role for filopodia in haptotactic sensing; where increased ligand density promotes the binding of filopodia and therefore, the eventual direction of migration.

To obtain the quantitative data in the studies mentioned above, a host of image analysis methods were developed. Here we present some of the techniques we developed for analysis of fluorescence, differential interference contrast (DIC), and phase contrast images. These techniques primarily involve the segmentation of cytoskeletal or signaling structures/domains for the purpose of tracking, comparison, or quantification. We demonstrate analyses that utilize signals from particular fluorescent probes and methods that identify subcellular structures based on morphological criteria, these structures can be analyzed directly or masks of these structures can be used to spatiotemporally restrict subsequent signaling analyses. These techniques offer quantitative approaches for assessing signaling and cytoskeletal phenomena in migrating cells.
Cytoskeletal and Signaling Dynamics Underlying Directional Persistence of Cell Migration

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Chemical Engineering

Raleigh, North Carolina

2015

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DEDICATION

This dissertation is dedicated
to my family and friends
for all their love and support
BIOGRAPHY

Heath Ellis Johnson was born in August 1985 to Greg and Stacy Johnson in East Tennessee. He was raised just outside the town of Morristown, TN with his two sisters Courtney and Lena. Heath attended public school in Morristown and after completing it in 2003 he moved to Knoxville to attend the University of Tennessee. Heath debated between philosophy, geology, and several engineering and science majors before finally deciding to major in Chemical Engineering. During the last 2 years of his undergraduate career Heath was given the opportunity to participate in research and became interested in continuing it in his career. With the encouragement of one of his professors, Dr. Paul Frymier, he decided to addend graduate school to pursue a PhD. In the fall of 2008 Heath moved to Raleigh to attend North Carolina State University for his graduate studies where he worked under the direction of Dr. Jason Haugh on fibroblast migration. After completing his work here, Heath plans to continue research in a post-doctoral position.
ACKNOWLEDGMENTS

There are many people for whom I would like to acknowledge and thank for their support over time in graduate school. First and foremost, I would like to express my sincere thanks to my advisor Dr. Jason Haugh who has been a mentor and a friend to me. He took me into the group despite my lack of biology experience and has encouraged me ever since, giving me both freedom and guidance with my research. I could not have asked for a better advisor. Thanks to all my colleges in Haugh and Bear groups, present and former, for their support academically and at meal time – Dr. Erik Welf, Dr. Kyle Grant, Dr. Shoeb Ahmed, Dr. Samantha King, Dr. Sreeja Asokan, Dr. Keefe Chan, Dr. Jeremy Rotty, Xiaji (Astor) Liu, Anis Rahman, Toufiq Rahman, and Elizabeth Haynes. I would also like to express gratitude to the other members of my thesis Committee: Dr. Robert Kelly, Dr. Balaji Rao, and Dr. Glenn Walker for their direction and support. Additionally, from my pre-graduate school career, I would like to thank Dr. Paul Frymier, my undergraduate advisor as well as my high school English teacher, Marilyn Ware, for their advice and faith in me throughout my academic life.

I would like to thank all of my friends I have made in my time here at NC State; it would not have been the same without you. One of the kindest and hardest working people I have ever met - Stephanie Lam, thank you for all you have done for me. Philosopher, future professor, and legendary eater - Stefano Menegatti, thanks for all the great conversations and food. Josh McClure and Christina Devine-McClure were there from start of my time in grad school and have been there for me the whole time. Thanks to them and my other friends I’ve made here - Josh Allen, Dave Latshaw, Phillip Schoch, Alina Higham-Latshaw, Will Sweet,
Amit Mishra, and Joseph Eby – you guys made grad school a lot of fun! Special thanks for everyone who participated in my music videos in particular Josh McClure, Josh Allen, Craig Needham, and Timothy Shay.

Finally I would like to thank my entire family for their enduring support: My parents for raising and always supporting me, all of my grandparents, Virgie and Ellis for helping raise and feed me, Carolyn, Robert, and Milton, for their encouragement and support, my sibblings, Courtney and Lena, as well as my brothers by another mother, Dr. Terence Long and Keith Langston, my aunt Tesha and my cousins, and finally my uncle, Charles, for encouraging and sharing my interest in science when I was a kid.
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Figure 3.6. (a) Montage of PI3K signaling (pseudocolored) and fascin-1 localization (inverted grayscale) during protrusion over actin bundles in an NIH 3T3 cell, representative of 31 cells viewed. Scale bar = 10 μm. (b) NIH 3T3 cells co-expressing mCherry-AktPH and TFP as a volume marker were used for ratiometric analysis. Average enrichment ratios (mean ± 95% confidence interval) of PI3K signaling in filopodia (left) and the associated histograms (right) for untreated cells (blue, n = 724 filopodia) versus cells treated with 1 μM PI3K-α inhibitor IV (red, n = 119 filopodia) are shown. (c) Montage of mCherry-AktPH/TFP ratio (pseudocolored) showing PI3K signaling enrichment preceding lamellipodial protrusion over an actin bundle (left). Scale bar = 5 μm. For each cell, the percentage of
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**Figure 3.7.** (a) Montage of PI3K signaling (pseudocolored) and fascin-1 localization (inverted grayscale) during protrusion over actin bundles in an NIH 3T3 cell, representative of 31 cells viewed. Scale bar = 10 μm. (b) NIH 3T3 cells co-expressing mCherry-AktPH and TFP as a volume marker were used for ratiometric analysis. Average enrichment ratios (mean ± 95% confidence interval) of PI3K signaling in filopodia (left) and the associated histograms (right) for untreated cells (blue, n = 724 filopodia) versus cells treated with 1 μM PI3K-α inhibitor IV (red, n = 119 filopodia) are shown.

**Figure 3.8.** (a) Two-color TIRF imaging of zyxin and paxillin in NIH 3T3 cells confirms that mature, zyxin-containing adhesions are found some distance behind the leading edge. (b) Two-color TIRF imaging of fascin and zyxin in NIH 3T3 cells confirms that zyxin-containing adhesions are typically located behind F-actin bundles at the periphery. (c) TIRF images of FP-AktPH-expressing NIH 3T3 cells, plated on either fibronectin or poly-lysine, before and after inhibition of Src-family kinases (PP2, 10 μM). The images are representative of 13 cells viewed for fibronectin and 19 cells viewed for poly-lysine.

**Figure 3.9.** TIRF images of FP-AktPH-expressing NIH 3T3 cells, plated on either fibronectin or poly-lysine, before and after FAK inhibition (10 μM FAK inhibitor II). For each cell, the after/before ratio compares the difference between the mean intensity of the morphological extension with the highest intensity and the mean intensity of the center region of the contact area; a value significantly less than 1 indicates that the pattern became more uniform after FAK inhibition. Values are reported as mean ± 95% confidence interval for the fibronectin (n = 19) and poly-lysine (n = 24) data.

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Figure 3.11. (a) Two-color TIRF imaging of Lifeact and paxillin before and after inhibition of Arp2/3 complex by 100 μM CK666. The arrows show where small adhesions were lost following collapse of lamellipodia. (b) Same as (a) but with inhibition of PI3K (1 μM PI3K-α inhibitor IV). (c) Two-color TIRF imaging of AktPH or Lifeact biosensor with tagged paxillin in cells depleted of fascin-1. (d) Left: Kymograph of protrusion width during periodic protrusion waves as in Figure 3.12. Lines are drawn through the waves and used to calculate the velocity of each wave. Right: Histogram indicating velocities of protrusion dilation waves (n = 48), estimated from kymograph as shown on the left. These were taken from high-resolution videos of 11 recurrent protrusions observed in 6 different cells.

Figure 3.12. Protrusion dilation waves as shown by pseudocolored mCherry-AktPH (left) representative of 11 such sequences viewed in 6 cells. Scale bar = 10 μm. The protrusion width and FP-AktPH intensity are plotted for the same sequence on kymograph-like plots (right).

Figure 3.13. (a) TIRF montage, representative of 13 cells, showing filopodia-templated protrusion in IA32 MEFs co-expressing EGFP-fascin (inverted grayscale) and mCherry Akt-PH (pseudocolored). Scale bar = 10 μm. In the example shown, protrusions over filopodia coalesce to form a broad lamellipod. (b) Representative immunoblot showing relative fascin-1 expression levels in the non-targeting shRNA, fascin shRNA (#1), and fascin rescue IA32 cells used in directed migration experiments. (c) Chemotaxis assay results: wind-rose plots showing the distributions of overall cell migration directionality, expressed as an angle relative to an external gradient of PDGF, are shown for control (n = 74) and fascin-1 shRNA (n = 131). Mean FMI, velocity and persistence (D/T) are displayed +/- 95% confidence intervals. (d) Haptotaxis assay results are shown for control (n = 128), fascin-1 shRNA (n = 124), and fascin rescue (n = 57) tracks. Mean FMI, velocity and persistence (D/T) are displayed +/- 95% confidence intervals.

Figure 3.14. Conceptual model of morphodynamics directed by F-actin bundles. Peripheral actin bundles, e.g. filopodia, template clustering of nascent adhesions and harbor PI3K signaling. The bundles direct lamellipodia, formed by Arp2/3-mediated actin polymerization, by setting the preferred sites and directions of protrusion initiation and
propagation, respectively. An adhesion-based positive feedback loop (inset) amplifies PI3K signaling as the lamellipod spreads, promoting additional activation of Arp2/3 complex and formation of new actin bundles. The new bundles can direct initiation of distinct lamellipodia, seemingly branched from existing ones. Propagation of lamellipodia is self-limiting or metastable, and therefore protrusion and PI3K signaling cease, and the lamellipodia at least partially retract but typically leave the F-actin bundles intact. Hence, the cycle can repeat, sometimes as a series of traveling protrusion waves...

Figure 4.1. Basic image processing and segmentation. a) Unprocessed TIRF image of randomly migrating NIH 3T3 fibroblasts expressing GFP-AktPH. Scale bar = 20 μm. b) Cropped, rescaled, and pseudocolored image from a. The image has been cropped to contain the migration path of a single cell throughout the time course. The dark square in the top left corner of the field shows where another cell entering the region at a later time has been removed. Scale bar = 20 μm. c) Above: mask of the cell in b obtained by identifying the first bin of the k-means segmentation as the background. Below: mask of the cell (blue) with hot spot regions overlayed (red).

Figure 4.2. Advanced image analysis methods. a) Construction of the macroscopic signaling vector (SR) as outlined 4.2.3. If the goal is to correlate SR with the centroid movement vector, C, averaging over larger time intervals may be warranted, depending on the temporal resolution of the movie (Δt) in relation to the cell movement speed. b) Hot spots were tracked from birth to death as outlined in 4.2.4. Their centroid paths are plotted as connected lines, with elapsed time color-coded from cool to warm. c) Montage of the cell depicted in Fig. 1 (left) and the accompanying spatiotemporal signaling map (right), created as in 4.2.5. Times shown on the montage correspond with those on the map. Scale bar = 20 μm.

Figure 4.3. Automated identification of filopodia. (a) Putative filopodia are segmented (red; cell mask shown in yellow) by top-hat filtering of TIRF images. These structures may be tracked based on frame-to-frame overlap/proximity. (b) The structures thusly identified tend to be enriched in FP-tagged fascin-1. (c) The putative filopodia are labeled by FP-tagged Myosin X at their tips. Scale bars = 10 μm.

Figure 4.4. (a) Putative filopodia were identified from volume marker (TFP) images as described under section 4.2.6. The mask associated with filopodia is dilated, and the mean intensity of each filopodial region is
computed along with that of the cell interior. The ratio of those intensities (filopod/interior) is recorded. This procedure is then repeated for each region of pixels in the other channel, the mCherry-AktPH biosensor, for example. In this case, the enrichment ratio, $E$ (the filopod/interior ratio of AktPH normalized by that of the volume marker) is used to assess the presence of PI3K signaling in the filopodia. (b) The value of $E$ reflects both the extent of biosensor translocation (from the cytosol to the membrane) and the filopod geometry, effects that are not readily decoupled. Using a glycophasphatidylinositol (GPI)-anchored FP to uniformly label the outer leaflet of the plasma membrane, we estimated the relative enrichment of plasma membrane area relative to the volume of a filopodium. With FP-GPI in place of FP-AktPH, the mean value of $E$ was 4.7 (n = 798 filopodia from 9 cells; error bars indicate ± 95% confidence interval), indicating a significant effect of geometry. Put another way, the ratio of AktPH/volume marker TIRF should not be used to compare relative 3’ phosphoinositide densities in filopodia versus elsewhere in the contact area.

**Figure 4.5.** Examples of extension analysis to profile changes in cell morphology across subgroups. Analysis of extension breadth and total extended area was quantified for many cells for 300 timepoints over 2.5 hours. The analysis was performed for a LKB-1 null melanoma cell line as well as LKB-1 addback and MARK dominate negative version. Grey dots represent the mean values for individual cells over the timecourse. Blue lines ± standard deviation. Red boxes indicate 95% confidence intervals and the red lines indicate the mean.

**Figure 4.6.** (a) Enriched pixels in a chemotaxing cell are shown outlined in magenta. The intensity values of the segmented pixels are summed within angular bins at each time point to create a signaling “map” with the angle plotted on the horizontal axis and time on the vertical axis. Histogram showing the cumulative intracellular DAG distribution in chemotaxing cells expressing (C1)2-GFP (n = 12 cells). (b) Enriched pixels in a randomly migrating cell are shown outlined in magenta. The intensity values of the segmented pixels are summed within angular bins at each time point to create a signaling “map” with the angle plotted on the horizontal axis and time on the vertical axis. Histogram showing the cumulative intracellular DAG distribution in randomly migrating cells expressing (C1)2-GFP (n = 13 cells).
Figure 4.7. Method of analysis of the localization of DAG intensity in cells transfected with (C1)2-GFP. (Top) Original pseudocolored image is shown in the first panel followed by the cell mask, and peripheral region. (Bottom) Segmented enriched peripheral signaling, (bottom right) Signaling map from the cell shown. 

Figure 4.8. Arp2/3 complex edge ratio: (Top left) Cell stained for arp2/3 complex. (Top Right) High Arp2/3 complex signal designated as intensity greater than 1.2 standard deviations above the mean signal for the whole cell. (Bottom Left) High signal restricted to a 5 pixel band along the cell perimeter. (Bottom Right) Angular distribution of Arp2/3 enrichment along the perimeter of the cell.

Figure 4.9. (a) TIRF microscopy movies of chemotaxing MyoRLC-GFP cells are analyzed to identify puncta (blue) and stress fiber regions (red). The localization of puncta and stress fibers relative to the cell centroid were averaged across multiple cells (n = 11 cells) and presented as histograms. (b) Analysis of puncta and stress fiber distributions, quantified as in (A), in MyoRLC(S1AS2A)-GFP cells (n = 36 cells). (c) Circular histograms of MyoRLC-GFP (n = 162) and MyoRLC(S1AS2A) (n = 67) cells chemotaxing in a gradient of BLB. (d) Intensity of MyoRLC-GFP and MyoRLC(S1AS2A)-GFP during chemotaxis in a BLB gradient, presented as a histogram.

Figure 4.10(a) Image of a chemotaxing MLC-GFP cell. Magnified sections show myosin organization with puncta in the blue box and stress fibers in the red box. (b) A two-dimensional median filtering (1) is used to segment, puncta (2) and stress fibers (3) in the outer ring of the convex hull mask. Puncta are marked as blue and Stress fibers as red in the final segmentation (4). (c) The intensity values of the enriched segmented pixels in MyoRLC-GFP and MyoRLC(S1AS2A)-GFP cells chemotaxing to BLB gradient are summed within angular bins at each time point to create a signaling “map” with the angle plotted on the horizontal axis and time on the vertical axis.

Figure 4.11.(a) Accurate phase segmentation from higher resolution movies for morphological analysis. (b) Rough cell outlines for single cell centroid tracking from low resolution imaging data.

Figure 5.1. (a) An NIH 3T3 cells co-expressing: mCherry-AktPH (grayscale) and EGFP-MyoX (green) (left, representative of 15 cells in 4 experiments) exhibits normal morphology in the absence of fascin overexpression; when mCherry-fascin (grayscale) is expressed in
conjunction with EGFP-MyoX (green) (right, representative of 16 cells in 6 experiments) a morphology with exaggerated filopodia and almost no lamellipodia is produced. Scale bar = 20 μm.
CHAPTER 1*

Introduction to Cytoskeletal and Signaling Components of Mesenchymal Cell Migration

1.1. Introduction

The main objectives of my research are to (1) understand the role of the phosphoinositide 3-kinase (PI3K) signaling pathway in directed cell migration, (2) understand the mechanism(s) by which mesenchymal cells form and orient new protrusion structures called lamellipodia, and (3) develop image analysis techniques to aid quantification of signaling and morphological changes during cell migration. Understanding cell migration is critical to unraveling the bases for development, tumor metastasis, and immune responses. Cell migration is not simply about the speed of locomotion, but the ability to control when and how to efficiently change direction as well. Cells must sense and respond to complex external and internal signals and dynamically reorganize the cytoskeleton accordingly. Cell migration can be biased by external cues, such as chemical gradients, varying extracellular matrix (ECM) composition, and mechanical forces exerted upon the cell. Even in the absence of spatially biased external stimuli, many cell types exhibit spontaneous cytoskeletal organization and persistent migration with random directionality. However, in a “uniform” external environment cells are still subjected to molecular noise from the orientations and positions of individual molecules; furthermore, in a physiological setting a cell is never truly removed from external interferences by other cells, ECM variances, soluble factors, and physical stresses placed upon it. Interplay between the cytoskeleton and internal signaling cascades can direct this basal motility in the absence of external cues. Therefore, understanding the mechanisms that govern random migration is necessary to understand migration in complex, non-uniform environments. Critical to directed cell migration is the concept of directional persistence. Changes in direction of migration require reorientation of
the cytoskeleton, which is not rapidly achieved. Persistence can limit a cell’s ability to respond to cues rapidly but also helps keep cells on track once migration is established in the “correct” direction. Previous work from our lab (Weiger et al., 2010) showed that directional persistence of randomly migrating fibroblasts is spatiotemporally correlated with formation of regions of localized PI3K signaling. Hence we sought to unravel the mechanisms by which PI3K influences directional persistence versus turning behavior.

In this thesis, we investigate the cytoskeletal architecture that determines directionality and reorientation of mesenchymal cell migration, and we mechanistically link those cytoskeletal morphodynamics to PI3K signaling. In this Chapter, the distinct features of mesenchymal migration and the factors that bias it are introduced. The major underlying cytoskeletal and signaling processes known to affect cell migration, and the primary experimental techniques used to study them, are reviewed. In Chapter 2, we characterize the phenomenological mechanisms of fibroblast migration and the role of PI3K signaling. In Chapter 3, we elucidate the cytoskeletal and signaling components that govern those large-scale changes. In Chapter 4, image analysis methods that we developed to quantify these phenomena are presented. Finally, a summary of the work and future research directions is presented in Chapter 5.

1.2 Overview of Mesenchymal Cell Migration

1.2.1 Directed Cell Migration

While most cells migrate randomly in the absence of spatial cues, many forms of directionally biased migration are also possible, including haptotaxis, durotaxis, and
chemotaxis (Fig 1.1a). Haptotaxis is cell migration directed along a gradient of immobilized ECM, whereas durotaxis is migration directed by a gradient in mechanical stiffness. The aforementioned chemotaxis is characterized by three interrelated phenomena: the formation of periodic, self-organizing protrusions, directional sensing, and polarization (Van Haastert and Devreotes, 2004). Polarization is the propensity for cells to assume an asymmetric shape, characterized by localization of certain molecules at the leading and trailing edges (Devreotes and Janetopoulos, 2003). For example, protein assemblies such as the WAVE regulatory complex and F-actin are primarily found at the leading edge of the cell, whereas myosin II is prominent at the trailing edge. Chemotactic sensing requires a gradient of chemoattractant. The formation of pseudopodia is biased by signal transduction pathways such as activation of PI3K, mediated by chemoattractant receptors (Anand-Apte and Zetter, 1997).

One context of directed cell migration that will be discussed in more depth is wound healing (Fig. 1.1b). Shortly after wounding, platelet aggregation forms a hemostatic plug while the coagulation of blood forms a provisional matrix compromised of a cross-linked network of fibrin and fibronectin (Midwood et al., 2004). Chemoattractant gradients, together with the presence of the provisional matrix, act to recruit neutrophils and macrophages into the wound site in order to prevent infection and remove debris from the wound. Over the next few days, chemoattractants such as platelet derived growth factor (PDGF) are released, causing fibroblasts to migrate into the wound where they replace the provisional matrix with granulation tissue rich in collagen and fibronectin. Both the
provisional matrix and granulation tissue provide an extracellular matrix (ECM) for cell adhesion and migration.

For the purposes of this study, the context of fibroblasts chemotaxis biased by a PDGF gradient, as seen during wound healing, will be considered. Fibroblasts, like other eukaryotic cells, sense chemoattractant gradients spatially, directing movement by biasing local actin and/or myosin II dynamics. Fibroblast migration in response to a PDGF gradient is distinct from other cell types in that fibroblasts do not have the ability to directly amplify shallow gradients at least at the second messenger level as amoeboid cells can. They also require steep PDGF gradients of greater than a 10% difference in concentration across the cell in order to bias migration.

In addition to stimuli that guide cells to places necessary for physiological processes, spatial cues can also guide tumor cells to metastasize. Dense matrix can build up around tumors, potentially creating a durotactic cue and guiding cells from the tumor into the vasculature (Fig 1.1c). Other cues elsewhere in the body can then direct those cells to invade other tissues (metastasis), forming new tumors.
Figure 1.1. Directed migration cues for mesenchymal cells. (a) Diagram illustrating the diverse types of directional cues that mesenchymal cells respond to. Of note is the hybrid cue where chemotactic cues (e.g., growth factors) are bound to ECM scaffolds. (b) During cutaneous wound healing, fibroblasts (prototypical mesenchymal cells) respond to both PDGF (chemotaxis) and ECM cues (haptotaxis/durotaxis). (c) Likewise, mesenchymal tumor cells emerging from primary tumors sense multiple directional cues. Reprinted from (Bear and Haugh, 2014) with permission.
1.2.2. Distinct features of Mesenchymal Migration

Mesenchymal migration is structurally and biochemically distinct from amoeboid migration in several ways. Mesenchymal migration is characterized by strong adhesive forces used to generate traction at the leading edge of the cell. Conversely amoeboid migration relies on squeezing forces on the sides and rear of the cell to push forward. Amoeboid cells also exhibit increased polarity when compared to mesenchymal cells, which typically have multiple competing lamellipodia instead of a single large lamellipodia (Welf et al., 2012). The chemoattractant receptors of amoeboid cells are typically G protein coupled receptors (GPCRs) as opposed to those in mesenchymal cells which are typically receptor tyrosine kinases (RTKs). While these both activate similar pathways, the regulation of these pathways differs, and GPCR signaling is subject to receptor desensitization that RTK signaling is not (Bear and Haugh, 2014). In addition to migrating as single cells, some cell types are capable of migrating collectively, in which large groups cells move together as sheets or strands (Friedl and Gilmour, 2009). Cells can transition between these various migration modes or adopt one of these modes directly from a non-migratory state, such as epithelial cancer cells undergoing an epithelial-to-mesenchymal transition (EMT) prior to invasion (Thiery, 2002). A number of factors including the cell type, cell-cell interactions and composition and physical properties of the ECM play a role in determining the mode of migration used (Friedl, 2004).
Figure 1.2. Mesenchymal vs. amoeboid motility and chemotaxis. The illustrations and table compare the structural and dynamic features of mesenchymal migration with those of amoeboid cells such as neutrophils and lymphocytes. Reprinted with permission (Bear and Haugh, 2014).
1.3 Cytoskeletal Structures and Signaling Pathways in Cell Migration

1.3.1. Actin Structures in Cell Migration

The standard model of cell migration occurs through four basic steps: protrusion of the leading edge, adhesion to the ECM, cell body translocation, and retraction of the trailing edge as shown in Figure 1.3 (Mattila and Lappalainen, 2008; Ridley et al., 2003). In reality, many of processes may occur simultaneously; though in all of these processes, various actin structures play important roles. Two important organelles are primarily comprised of actin, lamellipodia and filopodia. Lamellipodia are thin, 0.1-0.2 micrometers thick, sheet like protrusions that contain branched networks of actin. Filopodia are also thin (0.1-0.3 micrometers) structures but are made up of tight parallel bundles of filamentous actin and have a finger-like shape (Mattila and Lappalainen, 2008). Although the role of filopodia is not completely understood, they are thought to act as “antennae” to probe the environment. Filopodia promote cell motility and contain receptors that can interact with the ECM and form the initial focal adhesion sites with it (Galbraith et al., 2007). Focal adhesion complex components, such as paxillin, are then recruited to form mature focal adhesions. Formation of focal adhesions is perpetuated by force produced by stress fibers (Senju and Miyata, 2009). Stress fibers are composed of antiparallel actin filaments that are associated with myosin II bundles, motor proteins which provide the contractile force that is necessary for migration (Mattila and Lappalainen, 2008).
Figure 1.3 Cell Migration is Dependent on Different Actin Filament Structures. (a) Motility is initiated by an actin-dependent protrusion of the leading edge, which is composed of lamellipodia and filopodia. These protrusive structures contain actin filaments, with elongating barbed ends orientated towards the plasma membrane. (b) During cellular extension, new adhesions with the substratum are formed under the leading edge. (c) Next, the nucleus and the cell body are translocated forward through actomyosin-based contraction forces that might be mediated by focal adhesion-linked stress fibers, which also mediate the attachment to the substratum. (d) Then, retraction fibers pull the rear of the cell forward, adhesions at the rear of the cell disassemble and the trailing edge retracts. Reprinted from (Mattila and Lappalainen, 2008), used with permission.
The polymerization of actin drives protrusions in the form of lamellipodia and filopodia at the leading edge of the cell. Actin filaments are polar structures, each possessing a rapidly growing end called the barbed end and a slow growing end called the pointed end. The barbed ends of actin are oriented towards the plasma membrane of the cell (Pollard et al., 2000). New actin filaments can be readily added to barbed ends to extend filaments. Capping proteins can inhibit this process by blocking barbed ends preventing new filaments from being added (Pollard, 2007). Alternatively, a mutli-protein complex, Arp2/3 can bind to the filaments allowing formation of a branched filament at 70° relative to the original filament (Rouiller et al., 2008).

Arp2/3 is thought to be primarily responsible for procuring the branched actin networks in lamellipodia. However, Arp2/3 nucleated branched filaments can converge to nucleate filopodia (Korobova and Svitkina, 2008; Svitkina et al., 2003) (Figure 1.4). BAR domain proteins such as IRSp53 are thought to promote membrane curvature and enhance protrusion. The actin filaments can be bundled together by proteins such as Fascin. Actin bundles become filopodia as they push forward ahead of the lamellipodium. Formins such as Dia2 prevent capping of these growing filament bundles and proteins like Ena/VASP deliver new actin filaments to the ends of filopodia, promoting their growth (Breitsprecher et al., 2011). Myosin X can transport primed integrins to the tips of the filopod, promoting attachment to ECM ligands.
Figure 1.4 Model describing the functions of key proteins at different stages during filopodia formation. However, it is important to note that some controversies exist concerning the activities of individual proteins, and it is likely that the relative importance of the filopodial components varies between different organisms and their cell types. (a) A subset of uncapped actin filaments of the actin-related protein-2/3 (ARP2/3)-nucleated dendritic network are targeted for continued elongation by the formin Dia2 (diaphanous-related formin-2) and/or by ENA/VASP (enabled/vasodilator-stimulated phosphoprotein) proteins. Dia2 also nucleates the formation of new, unbranched actin filaments. The barbed ends of these elongating actin filaments are converged together through the motor activity of myosin-X, leading to the initiation of a filopodium. (b) When the preliminary filopodium begins to push the plasma membrane, insulin-receptor substrate p53 (IRSp53) (or other inverse (I)-BAR domain-containing proteins) might further facilitate plasma membrane protrusion by directly deforming or tubulating the membrane. Alternatively, IRSp53 might sense the negative membrane curvature that is induced by pushing forces of elongating filaments and recruit other components to the site of filopodial initiation. ENA/VASP proteins can also function as initial F-actin-crosslinking proteins in the tip of an elongating filopodium. (c) The incorporation of the actin crosslinking protein fascin in the shaft of the filopodium generates a stiff actin filament bundle. At this stage, myosin-X might localize adhesion molecules to the filopodium tip by processive barbed-end directed movement (arrow) and/or attach the elongating actin filament barbed ends to the plasma membrane through its interaction with phosphatidylinositol phosphates. Dia2 is localized in the 'tip complex' and controls the barbed-end elongation of the filaments. Reprinted with permission from (Mattila and Lappalainen, 2008).
Integrins are cell surface receptors which bind specific ECM ligands leading the formation of adhesion complexes with the substrate. Each integrin is made up of a heterodimer consisting of an α and a β subunit. In mammals a total of 26 (18 α and 8 β) subunits are known to exist (Johnson et al., 2009). These subunits dictate the specificity of an integrin for various ECM ligands such as fibronectin, collagens, and laminins. Fibroblasts express several integrin subunits though the β1 integrin is among the most heavily studied and thought to be critical for responses to ECM proteins such as fibronectin and collagen (Huveneers et al., 2008; Liu et al., 2010; Tian et al., 2002). Integrins can adopt both active and inactive conformation; these conformational changes are mediated by inside-out activation by molecules such as talin (Barczyk et al., 2010). Binding of integrins to ECM ligands allows the clustering of integrins and the formation of focal adhesions, the formation of which is allows for the mechanical force necessary for migration to be transduced and activates myriad of signaling pathways implicated in migration.

1.3.2. Rho-Family GTPases in Cell Migration

The formation of these actin filament structures is governed by certain biochemical pathways within the cell. Rac is thought to be a key regulator of cell migration and is known to induce rapid actin polymerization resulting in protrusion and membrane ruffling as well as the formation of focal adhesion complexes (Parri and Chiarugi, 2010). Rac is a monomeric guanosine triphosphate binding protein (GTPase) of the Rho protein family, which includes twenty intracellular signaling molecules in mammals (Heasman and Ridley, 2008; Ridley, 2001). The most highly conserved and most characterized Rho GTPases are Rac, Cdc42, and
RhoA. Like all GTPases, these proteins act as molecular switches to control cellular processes by cycling between an active GTP-bound state and an inactive guanosine diphosphate (GDP) bound state (Abdul-Manan et al., 1999; Heasman and Ridley, 2008). Guanine nucleotide-exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide-dissociation inhibitors (GDIs) regulate the cycling between the active and inactive states (Fig. 1.5) (Itoh et al., 2002). GEFs catalyze the removal of GDP and the binding of GTP, promoting the active state. GAPs promote hydrolysis of GTP to GDP, removing an inorganic phosphate and reverting the GTPase to its inactive state. Whereas active Rho-family GTPases are predominantly localized at the plasma membrane, the inactive form is sequestered in the cytosol by GDIs (Schmidt and Hall, 2002).

Rac is thought to be activated by a number of pathways including those involving PI3K, and Cdc42 as well as through integrins. Cdc42 can also induce actin polymerization, leading to formation of filopodia or shorter cell protrusions called microspikes (Kozma et al., 1995). RhoA also plays a role in actin dynamics and is thought to be mutually antagonistic with Rac (Nobes and Hall, 1995). Activated RhoA is thought to be responsible for the formation of stress fibers and the maturation of focal adhesion complexes (Larsen et al., 2003).
Though now commonly referred to as Rac, it was originally named Ras-related C3 botulinum toxin substrate because of the ability of botulinum toxin C3 to ADP-ribosylate it (Didsbury et al., 1989). This is not surprising because ribosylation is enhanced by GDP and GTP, hinting that Rac is a GTPase. The Rac subfamily includes Rac1, Rac2, Rac3, and RhoG. Each of the three isoforms holds different levels of importance depending on the cell type. For example, in fibroblasts Rac1 is required for lamellipodia formation, but in macrophages it is not necessary for migration (Wells et al., 2004; Wheeler et al., 2006). More recent studies have shown that Rac is not required for migration in a number of other contexts, despite its critical role in the creation of new lamellipodia (Wu et al., 2012).

GTP-bound Rac can activate a large number of targets including Cdc42, Wiskott-Aldrich syndrome protein (WASP) family Verprolin-homologous protein (WAVE), insulin receptor substrate p53 (IRSp53), mDia2, and p21 activating kinase (PAK) (Krause and Gautreau, 2014). In turn, these all have downstream targets that mediate the formation of lamellipodia, filopodia, and focal adhesions (Figure 1.5). Rac, Cdc42, and RhoA can all be activated through signaling mediated by integrins, the cell surface receptors that physically link the cell to the ECM, and through signaling mediated by chemoattractant receptors during chemotaxis.
**Figure 1.5:** Simple model of Rac signaling. Both Rac GEFs and PI3K can be activated through integrins or through growth factor receptors. GEFs promote the removal of GDP from Rac, allowing it to bind to GTP. Conversely, GAPs activate the catalytic ability of the GTPase and hydrolase GTP to GDP, inactivating the signaling ability of Rac. Active Rac can activate PAK and the WAVE complex leading to the formation of lamellipodia.
Three isoforms of WAVE exist in mammals: WAVE1, WAVE2, and WAVE3, and whereas the biochemical differences have been characterized, their functional differences are still up for debate. Recombinant or overexpressed WAVE is constitutively active, and is thought to be in a complex with several other proteins that inhibits its activity in vivo. Although a number of theories have been proposed, the precise nature of this regulation is not well understood, and many indications are contradictory (Abou-Kheir et al., 2008; Eden et al., 2002; Ismail et al., 2009; Oikawa et al., 2004; Takenawa and Miki, 2001). Some studies suggest that Rac binds to Sra1, a member of the complex, activating it but without causing disassembly of the complex (Ismail et al., 2009). Another suggests that IRSp53 binding to Rac is necessary for activation of the complex (Abou-Kheir et al., 2008). Whatever the mechanism, it is thought that Rac mediates activation of WASP, followed by Arp2/3 activation and nucleation of actin filaments.

Given that integrins activate Rac, together with the effect of active Rac on protrusion, suggest a Rac-mediated positive feedback loop (Figure 1.6). Integrin-mediated Rac activation occurs through two GEFs, βPIX and DOCK180, each recruited by paxillin, and thus localized to focal adhesions (Nayal et al., 2006; Para et al., 2009; Smith et al., 2008). Focal adhesion kinase (FAK) phosphorylates paxillin to create a docking site for CrkII adaptor, which in turn binds DOCK180. Similarly, PAK phosphorylation of paxillin allows recruitment of the scaffold GIT1 and its binding partner βPIX, leading to Rac activation. In the latter mechanism, PAK can presumably also phosphorylate nearby paxillin molecules once bound to GIT1 and Rac, allowing for more spatially localized feedback.
Figure 1.6: Two Mechanisms for Rac Positive Feedback. Both: Rac activation leads to increased protrusion velocity causing the formation of nascent adhesions which recruit paxillin. These adhesions can turnover or form more stable adhesions which in turn, inhibit protrusion. Left: Paxillin is phosphorylated by focal adhesion kinase (FAK) allowing the CrkII adaptor to bind followed by recruitment of the Rac-GEF DOCK180. Right: Phosphorylation of paxillin at a site separate from the one mentioned above enables binding of the scaffold GIT1, allowing for binding and activation of the Rac-GEF βPIX. This feedback is enhanced by the additional fact that PAK can be activated by Rac, after which it can bind GIT1 enabling it to phosphorylate additional paxillin molecules. Adapted from (Cirit et al., 2010; Welf and Haugh, 2010). Reprinted with Permission.

The Cdc42 downstream target WASP is analogous to the interaction between Rac-GTP and WAVE complex. As its name implies, WASP was first discovered as the product of the gene mutated in patients with Wiskott-Aldrich syndrome. Five forms of WASP exist in mammals, two of which can bind directly to activated Cdc42: WASP and N-WASP (Bompard and Caron, 2004). WASP and N-WASP are structurally similar, differing only by
an additional Verprolin (V) domain in N-WASP, though activation of N-WASP has been shown to result in polymerization of actin at a rate more than four times faster than activated WASP (Zalevsky et al., 2001). Unlike WAVE activation mediated by Rac, WASP binds directly to Cdc42-GTP as it contains a Cdc42/Rac interactive binding (CRIB) domain, causing WASP to adopt an open, active conformation that allows it to bind the Arp2/3 complex (Hoffman and Cerione, 2000). Binding of WASP/N-WASP to Arp2/3 promotes polymerization of actin and creation of protrusion structures, typically in the form of filopodia (Higgs and Pollard, 2001). Cdc42 has generally been thought to control polarity, and Rac clearly has some role in this too.

RhoA also plays an important role in cytoskeleton regulation, yet there is some debate about the nature of this role. Rac and Cdc42 are thought to reside at the leading edge of the cell, while RhoA was thought to be localized primarily at the rear of the cell, but this has become a subject of debate (Machacek et al., 2009). RhoA has traditionally been thought to antagonize Rac and vice versa, and RhoA was implicated in retraction instead of protrusion (Nimnual et al., 2003; Sander et al., 1999). This might be attributed to the fact that retraction is typically required for protrusion elsewhere in the cell to relieve the tension necessary for the cell to move forward. RhoA can activate Rho-associating coiled-coil forming kinase (ROCK) as well as mammalian homolog of Drosophila diaphanous (mDia) (Ishizaki et al., 1996; Leung et al., 1995; Matsui et al., 1996). ROCK can both activate myosin and inactivate myosin phosphatase by phosphorylation. Myosin is necessary for contractile force and promotes retraction. In contrast, mDia catalyzes actin polymerization, complicating the role of RhoA in cell motility.
As mentioned previously, crosstalk between Rac and other small Rho GTPases is thought to occur. The exact nature of crosstalk between Rho GTPases is not completely understood, though there seems to be several candidate mechanisms. Rho GDI can bind with most Rho GTPases causing competition between them (Boulter et al., 2010). Cdc42 and Rac can both activate PAK and can both be activated by isoforms of PIX (Manser et al., 1998). Rac is also thought to activate Cdc42 more directly but the mechanism is unclear (Nobes and Hall, 1995). Abi1 has been implicated in increasing both WASP and WAVE activity by forming complexes with them (Innocenti et al., 2005). In addition, Rac was originally thought to activate RhoA, but as of more recently the relationship appears to be mutually antagonistic as outlined above (Nimnual et al., 2003; Ridley et al., 1992).

1.3.3 Phosphoinositide 3-kinase

In addition to activation by integrins, Rac is thought to be activated downstream of phosphatidylinositol 3-kinase (PI3K) (Merlot and Firtel, 2003). PI3K has been cited as being important for many cellular processes including cell growth, proliferation, differentiation, and motility (Vivanco and Sawyers, 2002). Class I phosphoinositide 3-kinases (PI3Ks) affect various cellular processes ranging from metabolism to cell migration (Vanhaesebroeck et al., 2010). Excessive PI3K signaling is strongly linked to cancer progression and recalcitrance to anti-tumor drugs, therefore, PI3Ks are considered attractive targets for combination therapy (Wong et al., 2010). Class I PI3Ks are heterodimeric lipid kinases that catalyze phosphorylation of the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$] on the D3 position of the inositol ring to produce
phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P₃]. Each PI3K dimer comprises the catalytic subunit and a regulatory subunit that allosterically regulates the enzyme and targets the dimer to complexes with receptors and other signaling proteins at the plasma membrane. Class IA and 1B PI3Ks are distinguished by the binding specificities of their regulatory subunits. Class IA regulatory subunits contain Src homology 2 (SH2) domains that target the heterodimer to complexes containing certain tyrosine-phosphorylated proteins, whereas class IB regulatory subunits are recruited by Gβγ complexes liberated upon activation of heterotrimeric G-proteins (Hawkins et al., 2006). Class I PI3K activity is also mobilized by constitutively active mutants of the small GTPase Ras, which have a high incidence in certain cancers (Ramjaun and Downward, 2007). The phosphorylation reaction catalyzed by class I PI3Ks is reversed by the phosphatase and tensin homolog (PTEN) and other phosphoinositide 3-phosphatases, and loss of PTEN expression in cancer cells results in constitutively high PI(3,4,5)P₃ levels and excessive signaling (Song et al., 2012). The other major flux of PI(3,4,5)P₃ dephosphorylation is at the D5 position, which is catalyzed by Src homology 2 domain-containing inositol 5-phosphatases (SHIPs), a reaction that produces PI(3,4)P₂ (Tolias and Cantley, 1999). PI(3,4,5)P₃ and PI(3,4)P₂ engender both distinct and overlapping signaling functions through interactions with pleckstrin homology (PH) and phox homology (PX) domains found in many proteins (Lemmon, 2008).
1.4 Overview of Experimental Techniques

1.4.1 Total Internal Reflection Fluorescence Microscopy

In the early 1980’s, Axelrod and colleagues popularized the development of prism-based TIRF microscopes and their application to study fluorescently labeled proteins (Burghardt and Axelrod, 1981; Thompson et al., 1981) and cell membranes (Axelrod, 1981). Adoption of the technique in cell biology has steadily increased ever since. TIRF microscopy relies on the optical principle that propagating laser light is reflected at an interface adjoining a medium of lower refractive index when the angle of incidence exceeds the critical angle defined by Snell’s law. The wave theory of light further explains that the incident light does impart energy to the adjoining medium, but rather than propagating as a sinusoidal wave (as in light refraction), the energy decays exponentially as a so-called evanescent wave. The dynamic length scale of its decay is a fraction of the incident light wavelength; therefore, the length scale of decay is ~100 nm for visible light. According to these physical principles, the evanescent wave selectively excites fluorophores proximal to the interface, making TIRF microscopy ideal for high-resolution studies of dynamic processes that occur at or near the plasma membrane-substratum contact area in live, eukaryotic cells (Axelrod, 2001; Steyer and Almers, 2001; Toomre and Manstein, 2001). Early events in signal transduction are among those processes readily interrogated by live-cell TIRF microscopy, and its use to study PI3K signaling and other signaling pathways by Meyer and colleagues predated the availability of commercial TIRF microscopes (Codazzi et al., 2001; Haugh et al., 2000). For well-spread cells, TIRF illumination thusly yields a
crisper image and, more importantly for quantitative studies, an order-of-magnitude greater sensitivity relative to other modalities.

In this section we review some basic theory that is relevant to the interpretation of TIRF images. Laser light propagates in medium 1 (e.g., glass) and encounters an interface adjoining a medium 2 (e.g., aqueous buffer), which has a lower refractive index. We define the angle of incidence, measured from the inward normal of the interface, as \( \theta_i \) (i.e., the closer to 90°, the more “glancing” is the approach). To achieve total internal reflection, \( \theta_i \) must exceed the critical angle, \( \theta_c \), defined by Snell’s law.

\[
\theta_c = \sin^{-1} \frac{n_2}{n_1}
\]

The two parameters \( n_1 \) and \( n_2 \) are the refractive indices of mediums 1 and 2, respectively. The deviation of \( \theta_i \) from \( \theta_c \) determines the length scale of evanescent decay, \( d \), relative to the wavelength of the incident light, \( \lambda \).

\[
d = \frac{\lambda}{4 \pi (n_1^2 \sin^2 \theta_i - n_2^2)^{1/2}}
\]

For \( \lambda \) in the visible light spectrum and typical parameters \( n_1 = 1.52 \) for glass, \( n_2 = 1.33 \) for water, and \( \theta_i \) within a few degrees of 70°, \( d \) is calculated to be in the range of ~50-125 nm. Correcting for the non-uniformity of the refractive index as the evanescent wave penetrates through buffer, the plasma membrane, and cytoplasm (Reichert and Truskey, 1990) yields a somewhat higher estimated range for \( d \). Nevertheless, its value is an order of magnitude lower than the height of a typical mammalian cell.
Suppose that one is using TIRF to image a fluorophore with local concentration $C(x,y,z)$, where the $z$ coordinate is the height above the interface. If the refractive index of the sample medium were uniform (acknowledging that this is just a first-order approximation), the projected fluorescence intensity, $F(x,y)$, is formulated as an integral over $z$ as follows.

$$F(x,y) = \alpha \int_0^\infty C(x,y,z) e^{-z/d} dz$$

In the equation above, the parameters are $d$, the length scale of evanescent wave decay, and $\alpha$, a proportionality constant that accounts for the power density imparted at the interface, the excitation/emission properties of the fluorophore, and the quantum efficiency of the camera. An additional integration with respect to time, depending on the exposure interval, is implied.

For an intracellular biosensor, the equation above illustrates that the local TIRF intensity depends not only on the density of fluorescent biosensor bound at the plasma membrane, but also on the distance of the plasma membrane from the interface (offset by the cell glycocalyx and integral membrane proteins in contact with the surface) and the contribution of biosensor molecules in the membrane-proximal region of the cytoplasm. Thus, temporal changes or spatial gradients in fluorescence intensity, especially subtle ones, can be artifacts caused by undulation of the plasma membrane or depletion of the biosensor from the cytosolic pool. If one has access to multi-color TIRF, a cytosolic volume marker may be used to normalize for non-uniformities in membrane topography. A plasma membrane-targeted marker may also be used for this purpose, but these are subject to potential non-uniformities in membrane
composition and, especially if targeted to the inner leaflet of the membrane, could result in acute phototoxic effects.

1.4.2 Fluorescent Biosensors

To probe various signaling pathways, cells are transfected with a genetically encoded biosensor comprising a fluorescent protein, such as enhanced green fluorescent protein (GFP) or others with varying spectral properties fused to a modular domain that binds the target of interest. If the target is found on the membrane such as a lipid, binding results in translocation of the biosensor from the cytosol to the plasma membrane, which is monitored in real time. Although other modes of fluorescence microscopy may be used for this purpose, TIRF microscopy offers unequaled \( z \)-resolution of the cell-surface contact zone. Alternatively, full length versions of the molecule one wishes to observe can be fused with a fluorescent protein and translocation can be observed directly. The disadvantage of this technique is that it essentially requires overexpression of the protein which may have an effect on localization of the probe or the cells behavior.

When selecting a biosensor there are several properties that must be considered. For example, if choosing a PH domain biosensor, one should consider the specificity, affinity, and kinetics of its lipid binding, as well as the effective intracellular concentrations of the lipid target(s) and the expressed biosensor. Several PH domains, such as those of Bruton’s tyrosine kinase (BTK) and cytohesin-1, bind specifically and with different affinities to the lipid product of class I PI3Ks, PI(3,4,5)P\(_3\). Yet, for probing PI3K signaling we favor use of the PH domain derived from Akt-1 (AktPH), which has been fused to the C-termini of
various fluorescent proteins. AktPH binds equally well to both PI(3,4,5)P$_3$ and PI(3,4)P$_2$, and thus it is best suited for monitoring the phosphorylation and dephosphorylation of the D3 position. Further, it binds with moderate (high-nanomolar) affinity (Kavran et al., 1998) and sufficiently fast kinetics, properties that are critical for quantitative studies (Haugh, 2012). Although the binding affinity of AktPH is nearly optimal for translocation measurements, unnecessarily high biosensor expression can produce a dominant-negative effect and should be avoided (as discussed further in Appendix A.3). To verify the function of the AktPH fusion (or an alternate biosensor), cells can be tested for appropriate translocation responses to stimuli known to elicit PI3K activation, and it should be confirmed that translocation is prevented or reversed by PI3K inhibition, preferably by both pharmacological and genetic means.

A biosensor that does not require localization are Förster resonance energy transfer (FRET; sometimes also referred to as fluorescence resonance energy transfer) biosensors. FRET biosensors are not as simple to produce as translocation biosensors and require two different fluorophores which can both be on the same or separate molecules. Energy is transferred via long range dipole-dipole coupling from a fluorophore in an excited state, the donor, to another chromophore or acceptor (Jares-Erijman and Jovin, 2003). The ability of molecules to make this transfer is strongly dependent on the distance between the two fluorophores. This allows for FRET to only occur when the conformation of the probe changes in intramolecular FRET or when the two separate molecules bind each other in intermolecular FRET. In both cases, the probes must be specifically designed to put the fluorophores in very close proximity of each other upon binding. Unfortunately, the
efficiency of this transfer even in the most ideal cases is relatively low. To complicate things further, a pair of fluorophores which excitations and emissions overlap is required which usually also leads to some overlap of the emissions. Therefore the fluorescence of the acceptor molecule must be normalized by that of the donor as the contributions of FRET are often outweighed by bleed through of the donor into the acceptor channel.

1.5. References


CHAPTER 2’

Migrating Fibroblasts Reorient Directionality by a Metastable, PI3K-Dependent Mechanism

2.1. Introduction

Control of cell crawling behavior is fundamental to collective and concerted movements of multiple cell types, as seen during embryonic development and physiological responses to wounding and infection. To achieve productive migration, a cell must be spatially polarized, with differential localization of signaling, adhesion, and cytoskeletal processes to promote net protrusion at one end and net retraction at the other (Ridley et al., 2003). Maintenance of this fore-aft asymmetry is the basis for directional persistence, whereby a randomly migrating cell moves along fairly straight paths for sustained periods, punctuated by stochastic turning behavior that causes changes in orientation (Petrie et al., 2009). It follows that regulation of cell turning determines the fidelity of cell migration directed by chemotactic gradients and other dynamic and potentially competing spatial cues. Changes in direction should be suppressed while the chemotaxing cell is properly aligned with the gradient; otherwise, cell turning ought to be encouraged and biased so as to steer the cell in the proper direction.

During the past decade, directed cell migration has been characterized in two distinct ways that are, separately, best understood in the context of the chemotactic amoeba, *Dictyostelium discoideum*. One line of investigation concerns the polarization of intracellular signaling activities. Steep external gradients of cAMP elicit robust symmetry breaking, with Ras and phosphoinositide 3-kinase (PI3K) signaling localized with F-actin at the leading edge, in *D. discoideum* (Merlot and Firtel, 2003; van Haastert and Devreotes, 2004); characteristic of amoeboid cells, actin polymerization is balanced by squeezing forces mediated by myosin localized at the cell rear of the cell. Whereas early studies implicated
polarization of PI3K signaling in gradient sensing, it is now appreciated that its role is context-dependent, and that PI3K mediates only one of a few pathways known to be important for *D. discoideum* chemotaxis (Bosgraaf et al., 2008; Hoeller and Kay, 2007; Loovers et al., 2006; Swaney et al., 2010; Takeda et al., 2007). In the absence of a spatial cue, these pathways spontaneously polarize to govern random *D. discoideum* motility (Postma et al., 2003; Sasaki et al., 2007).

The other approach has been to characterize the morphological dynamics associated with leading edge protrusion. *D. discoideum* cells crawl by extending morphologically defined protrusions (pseudopods). Chemotaxing amoebae extend pseudopods with a characteristic frequency, with new pseudopods primarily branching from existing ones (Andrew and Insall, 2007). Directional persistence is maintained by extending pseudopods in an ordered manner, alternating between left and right of the cell migration axis (Bosgraaf and Van Haastert, 2009b). In the phenomenological model that has emerged, the cAMP gradient spatially biases an otherwise stochastic and excitable polarization process (Bosgraaf and Van Haastert, 2009a; Van Haastert, 2010; van Haastert and Postma, 2007; Xiong et al., 2010); however, even in this relatively well-characterized system, the connection between signaling and cell shape dynamics is presently unclear. cAMP stimulation elicits the formation of self-organizing domains in which PI3K signaling is locally enriched, and new pseudopods later emerge at those locations (Postma et al., 2003). In this context, however, inhibition of PI3K does not fundamentally alter pseudopod dynamics; it simply reduces the frequency of pseudopod generation (Andrew and Insall, 2007).
In contrast with cells that exhibit amoeboid movement, such as *D. discoideum* and leukocytes, fibroblasts and other mesenchymal cells are slow-moving and crawl by balancing actin polymerization and integrin-mediated adhesion dynamics at their leading edges (Friedl and Wolf, 2010; Vicente-Manzanares et al., 2009). During random migration, these cells often exhibit multiple, competing protrusions (lamellipodia) radiating in different directions, which has been linked to their migration behavior (Petrie et al., 2009). Fibroblasts with reduced expression of the Rho-family GTPase Rac1 are more elongated and move with greater directional persistence (Pankov et al., 2005), because cell protrusion and retraction are predominantly oriented along the migration axis. In another study, fibroblasts with muted expression of Rac1, Cdc42, and RhoG exhibited a similarly elongated morphology and a severe cell speed defect, but they oriented normally in a chemotactic (PDGF) gradient (Monypenny et al., 2009). On the time scale of seconds to minutes, the leading edge exhibits complex motility dynamics, including periodic protrusion/retraction switching (Abercrombie et al., 1970; Giannone et al., 2004) and lateral protrusion waves (Dobereiner et al., 2006; Machacek and Danuser, 2006; Weiner et al., 2007). Through the combined use of fluorescent biosensors and high-resolution image analysis, the spatiotemporal relationships between activation of Rho-family GTPases and such leading-edge morphodynamics have been elucidated (Machacek et al., 2009; Sabouri-Ghomi et al., 2008); however, given that the directionality of fibroblast migration is relatively long-lived, with estimated persistence times in the range of 20-70 minutes (Dunn and Brown, 1987; Gail and Boone, 1970; Ware et al., 1998), it is presently unclear how overall cell shape changes associated with reorientation/turning behaviors are coordinated at the level of intracellular signaling.
Here, spatiotemporal mapping of protrusion/retraction, PI3K signaling, and morphological dynamics in fibroblasts reveals that although membrane protrusion and recruitment of PI3K signaling are relatively short-lived, directional persistence is maintained by restricting where protrusion occurs. To achieve large-scale turns, migrating fibroblasts reorient migration polarity through branching and pivoting of lamellipodia. Inhibition of PI3K signaling blocks fibroblast reorientation by this mechanism, not by reducing the frequency of initiating new branches but rather their stability. Accordingly, localized PI3K signaling increases after, not before, the initiation of protrusion induced spontaneously or by liberation of photo-activatable Rac. Finally, it is shown that biasing the branch-and-pivot reorientation mechanism allows chemotactic fibroblasts to align migration directionality with the external gradient. We conclude that, unlike *D. discoideum* responding to cAMP, lamellipodial branching in fibroblasts is not a regular mechanism of motility but rather a stochastic process that resets migration polarity. The critical role of PI3K signaling in this process is not in the generation of new protrusions but rather in promoting lateral spreading and propagation of the branched state.

2.2. Results

2.2.1. Reorientation of Cell Migration by Coordination of Motility Dynamics Across Disparate Time Scales

We previously showed that PI3K signaling, monitored by total internal reflection fluorescence (TIRF) microscopy in migrating fibroblasts expressing the GFP-AktPH biosensor, is localized in protrusive structures during both random migration (Weiger et al.,
2010; Weiger et al., 2009) and chemotaxis (Melvin et al., 2011), and thus the pattern of PI3K signaling correlates with overall direction of cell migration (Fig. 2.1a). Furthermore, PI3K signaling is transient, with localized regions (hot spots) emerging and dying out with a characteristic time scale of ~15 minutes in randomly migrating cells; the dynamics are globally coupled, in the sense that the emergence of a hot spot tends to be shortly followed or preceded by the death of another (Weiger et al., 2010).
Figure 2.1. Reorientation of fibroblast migration by branch-and-pivot of protrusions. NIH 3T3 fibroblasts expressing GFP-AktPH were monitored by TIRF microscopy during random migration on fibronectin (n = 28). (a) Pseudo-color montage showing the characteristic branching and pivoting of protrusions and localization of PI3K signaling; scale bar = 20 µm. The sketch at right illustrates how protrusion velocity, mapped as a function of angular position and time, reveals branch-and-pivot behavior. (b) Spatiotemporal maps of protrusion (red)/retraction (blue) velocity, PI3K signaling hot spots, and morphological extensions for the cell depicted in a.

Here, for the same cohort of randomly migrating cells (n = 28), we mapped the radial protrusion/retraction velocity alongside the locations of PI3K signaling hot spots and regions of finger-like morphological extension as a function of angular position (relative to the cell
centroid) and time (Fig. 2.1b). These spatiotemporal maps reveal distinct dynamics on short and long time scales (minutes versus hours). Whereas individual protrusion and signaling events tend to be relatively short-lived, consistent with the previous analysis (Weiger et al., 2010), they are almost exclusively confined to long-lived morphological extensions of the cell. Thus, protrusion and retraction occur along well-defined tracks in the spatiotemporal map (Fig. 2.1b). Accordingly, across the cell population, PI3K signaling and protrusion are positively correlated, but the correlation of morphological extension with either protrusion or signaling is even greater (Fig. 2.2).
Figure 2.2. PI3K signaling, membrane protrusion, and regions of morphological extension are spatiotemporally correlated during random migration. Time-lagged correlations, pooling all angular positions, for the cohort of randomly migrating fibroblasts. From left to right, positive protrusion velocity relative to PI3K signaling localization, morphological extensions relative to protrusion, and morphological extensions relative to PI3K signaling are shown. Correlation coefficients were calculated for each cell, and the aggregate values are reported as mean ± 95% confidence interval (n = 28).

The dynamics of protrusion and retraction determine changes in cell shape and directionality. Whereas persistent protrusion at one end of a cell combined with retraction at the other end results in a smooth and straight migration path, deviation from that behavior causes cell reorientation. As illustrated in Fig. 2.1, cells execute dramatic turns by pivoting of protruding structures, characterized by a change in angular position with time, most often preceded by branching of a protrusion into two. Thus, if the two branches continue to extend symmetrically, the cell can achieve a turn of up to 90°. This appears to be a generic behavior exhibited by cells of mesenchymal origin; examples are found in time-lapse videos accompanying recent publications (Lo et al., 2004; Monypenny et al., 2009; Pankov et al., 2005; Uetrecht and Bear, 2009). Another characteristic behavior is switching of a region
between net protrusion, which is accompanied by intermittent PI3K signaling, and net retraction, during which PI3K signaling is silent. This behavior allows the cell to effectively reverse polarity and thus execute sharper changes in direction (Fig. 2.3).

**Figure 2.3.** Protrusion branching and switching between protrusion and retraction mediate sharp turns (n = 28 cells). A pseudo-color montage (scale bar = 20 µm) (a), contact area centroid path (b), and spatiotemporal map of PI3K signaling hot spots (c) show how abrupt changes in cell orientation correspond with changes in PI3K signaling (colored arrowheads).

2.2.2. **PI3K Signaling Promotes Cell Reorientation Through Dilation and Stabilization of Newly Branched Lamellipodia**

The preceding analysis suggests that the stochastic dynamics of PI3K signaling and protrusion are coupled to the longer-time-scale dynamics associated with cell turning. If so, interfering with PI3K signaling would be expected to alter turning behavior. Using a potent
pharmacological inhibitor with selectivity for type IA PI3Ks (PI3Kα inhibitor IV), titrated to a concentration (500 nM) that was just sufficient to almost fully inhibit PI3K signaling in most cells, we compared cell motility before and after addition of the drug (Fig. 2.4a). Strikingly, PI3K-inhibited cells adopt a more elongated morphology, with protrusion restricted to the poles. Although short-lived bifurcations were sometimes noticeable in the spatiotemporal protrusion map, stable branching and pivoting were virtually absent. The specificity of this effect was corroborated using a dominant-negative mutant of PI3K regulatory subunit p85α (Dhand et al., 1994); cells expressing this construct exhibited the same crawling phenotype as the drug-treated cells (Fig. 2.4b).
Figure 2.4. PI3K signaling is required for propagation but not initiation of branched protrusions. (a) Protrusion/retraction map and pseudo-color TIRF images (scale bar = 20 µm) of a randomly migrating fibroblast expressing GFP-AktPH; PI3Kα inhibitor IV (500 nM) was added at approximately the midway point. White arrowheads in the image mark sites of protrusion/retraction. The results are representative of 12 cells. (b) Protrusion/retraction map and pseudo-color TIRF image (scale bar = 20 µm) of a randomly migrating fibroblast co-expressing dominant-negative PI3K regulatory subunit and GFP-AktPH. The results are representative of 9 cells.

To examine the morphodynamics of branched protrusions in greater detail, we monitored fibroblasts co-expressing GFP-AktPH and tdTomato-Lifeact, a marker of F-actin,
during random migration; during the course of such experiments, PI3Kα inhibitor IV was sometimes added (Fig. 2.5a). Without PI3K inhibited, newly formed branches consistently become enriched in PI3K signaling and spread laterally (dilate) as they protrude, with a band of F-actin that broadens along the leading edge. Analysis of AktPH and Lifeact accumulation shows that these processes temporally overlap (Fig. 2.5b). With PI3K inhibited, nascent protrusions still form with regularity, but they fail to broaden and stabilize, and they almost invariably stall and eventually retract (Fig. 2.5a). Inspection of cells expressing dominant-negative PI3K regulatory subunit confirmed that nascent protrusions fail to dilate and are unstable when PI3K cannot be recruited (Fig. 2.5c).
Figure 2.5. (a) TIRF montage (scale bar = 10 µm) of a randomly migrating fibroblast expressing GFP-AktPH and tdTomato-Lifeact; PI3Kα inhibitor IV (500 nM) was added after approximately 3 hours. Arrowheads mark sites of protrusion with F-actin at the leading edge. The results are representative of 10 cells. (b) Line scan showing temporal overlap of AktPH and Lifeact accumulation in a transient protrusion. The TIRF images at right show the position of the line scan and correspond to the time indicated by arrowheads. (c) Nascent protrusions (arrowheads) are thin and short-lived in cells co-expressing dominant-negative PI3K regulatory subunit and GFP-AktPH.
Quantitative analysis revealed that inhibition of PI3K by either approach does not grossly affect the overall frequency of initiated branches, defined as the emergence of a protrusion in a distinct direction. Rather, PI3K inhibition prevents successful propagation of the branched state (Fig. 2.6a). To evaluate how the inability to branch and pivot impacts overall cell migration, motility metrics of control and dominant-negative p85-expressing cells were compared (Fig. 2.6b). PI3K inhibition did not significantly affect the overall migration speed or directional persistence (D/T ratio) of the cell population, whereas the PI3K-inhibited cohort showed reduced rates of protruded area generation and less sideways movement as judged by the ratio of elliptical axes of each cell’s migration path (Fig. 2.6b). The lack of effect on directional persistence was unexpected; retrospective analysis of the cell centroid tracks indicated that the PI3K-inhibited cells’ movements showed more back-and-forth reversals of direction that nonetheless lay along a nearly parallel path. Collectively, these results demonstrate that PI3K signaling, rather than serving as a prerequisite for protrusion per se, reinforces newly formed lamellipodia to promote large-scale turns in cell migration.
Figure 2.6. (a) Quantification of branch initiation and successful branch propagation (total number of events divided by total time) with versus without PI3K inhibition. The control cells \((n = 28)\) are the same as analyzed in Figure 2.1, DN p85 refers to cells expressing dominant-negative PI3K regulatory subunit \((n = 9)\), and the other cohorts are cells before and after treatment with PI3Kα inhibitor IV \((n = 12)\). (b) Cell motility metrics comparing the control and DN p85 cohorts (means ± 95% confidence intervals). Average cell centroid translocation speed, D/T ratio (direct distance from start to end divided by the total distance traveled), and average protruded area generation were evaluated for 12-minute intervals. The cell path axis ratio measures each cell’s degree of sideways movement relative to the major axis of migration.
Figure 2.7. PI3K signaling is localized after initiation of protrusion. (a) Pseudo-color montage illustrating local membrane protrusion followed by increases in GFP-AktPH localization in a randomly migrating fibroblast; scale bar = 5 μm. (b) Protrusion/retraction map for a representative cell migration experiment alongside a plot showing the time courses of positive protrusion velocity and PI3K signaling localization at the angular position marked by the black arrowhead.

2.2.3. PI3K Signaling is Localized After Initiation of Protrusion

To better define the relationship between local activation of PI3K signaling and leading edge protrusion, we sought to determine the temporal sequence of these two processes. Somewhat surprisingly, inspection of time-lapse images (Fig. 2.7a) and time series taken at fixed angular positions (Fig. 2.7b) revealed that localization of PI3K signaling tends to lag the onset of protrusion. Dual TIRF imaging of cells co-expressing mCherry-
AktPH and teal fluorescent protein confirmed that regions of AktPH accumulation are reasonably uniform in their apposition with the surface and that they do not introduce an artifact in locating the edge position (Fig. 2.8). Correlation of protrusion velocity and PI3K hot spot fluorescence with variable time lag (for all angular positions and cells) peaks with protrusion preceding signaling by ~1–2 minutes (Fig. 2.9a); the peak is sharpened considerably by correlating the positive derivatives (changes with respect to time) of the measurements, indicating that PI3K signaling increases in intensity after the movement of the leading edge begins to accelerate. Whereas PI3K signaling increases after initiation of protrusion, the reverse process — loss of PI3K signaling accompanied by net retraction — occurs with no perceptible time lag (Fig. 2.9a). Dual TIRF imaging of cells co-expressing mCherry-AktPH and GFP-paxillin, a marker of integrin-mediated adhesions (Laukaitis et al., 2001), shows that PI3K signaling increases during the transition of the adhesions from nascent to mature (Fig. 2.9b), underscoring the spatiotemporal coordination of signaling and adhesion dynamics in lamellipodia.
Figure 2.8. Soluble teal fluorescent protein controls for detection of PI3K signaling in lamellipodia and of leading-edge protrusion. mCherry-AktPH (mCh-AktPH)–expressing NIH 3T3 cells were cotransfected with cytosolic teal fluorescent protein (TFP) and monitored by TIRF microscopy. (a) Parallel pseudocolor images of mCherry-AktPH and teal fluorescent protein confirm that apparent PI3K hotspots are not an artifact of contact area topography. Bar, 20 μm. (b) The parallel TIRF images show the position of a linescan taken across the leading edge of a transient protrusion event. The kymograph below indicates the local rise in GFP-AktPH translocation after the initiation of protrusion. Comparison with the corresponding teal fluorescent protein kymograph shows that the position of the leading edge is not obscured by AktPH translocation. The arrowheads in the kymographs indicate the time corresponding to the snapshots above. Bar, 10 μm.
2.2.4. Protrusion Induced by Focally Activated Rac is Followed by Redistribution of PI3K Signaling

The results presented thus far suggest that PI3K signaling is not required for leading edge protrusion or maintenance of overall cell migration speed; rather, PI3K signaling is
mobilized after protrusion and subsequently promotes lateral spreading and propagation of the branched state. To further test this hypothesis, we used a fusion protein construct (PA-Rac) that enables reversible photo-activation of Rac signaling; by focusing blue-green light in a particular region of the cell, one can control the timing and location of Rac-induced protrusion (Wu et al., 2009). Indeed, focal activation of PA-Rac in cells co-expressing mCherry-AktPH catalyzed local protrusion from the sides of the cells (Fig. 2.10a). After initiation of protrusion, a clearly defined PI3K hot spot was formed at the site of photo-activation in 95% of the cells tested (n = 42); often, the hot spot emerged in tandem with reduction or disappearance of enriched PI3K signaling elsewhere (Fig. 2.10b), as expected based on the dynamic coupling of hot spots previously described (Weiger et al., 2010). Consistent with the results presented in Figure 2.6, PA-Rac-induced protrusion was equally robust in PI3K-inhibited cells (Fig. 2.11a). Finally, in support of the hypothesis that PI3K hot spot formation is associated with protrusion and not a by-product of supra-physiologic Rac signaling, PA-Rac failed to induce protrusion and hence did not dramatically alter the PI3K signaling pattern in cells with actin polymerization inhibited by addition of 200 nM cytochalasin D (Fig. 2.11b). Other experiments using cytochalasin D (Fig. 2.12) further support the notion that protrusion directs the dynamic redistribution of PI3K signaling but is not required for maintenance of PI3K hot spots in morphological extensions.
Figure 2.10. PI3K signaling is localized in response to protrusion induced by focally activated Rac. (a) Localization of mCherry-AktPH in fibroblasts co-expressing PA-Rac was monitored by TIRF microscopy, as shown in the pseudo-color montage (scale bar = 20 μm). Photo-activation of PA-Rac was initiated at the 18 min. mark in the region indicated by the red oval and was maintained there until after the 41 min. image shown. (b) For another cell, spatiotemporal maps of protrusion/retraction velocity and PI3K signaling localization show the typical patterns before, during (indicated by the dashed polygons), and after PA-Rac photo-activation. The results are representative of 42 cells.
Figure 2.11. (a) PA-Rac still elicits protrusion in cells treated with 1 μM PI3Kα inhibitor IV (scale bar = 20 μm). The results are representative of 6 cells treated with 0.5, 1, or 3 μM inhibitor. (b) Blocking protrusion by treatment with 200 nM cytochalasin D prior to photo-activation of PA-Rac prevents dramatic relocalization of PI3K signaling (scale bar = 20 μm). The results are representative of 11 cells thusly treated.
Inhibition of actin polymerization during random migration does not disrupt PI3K signaling. GFP-AktPH-expressing fibroblasts were monitored during random migration for ~8 h; at roughly the midway point, cytochalasin D was added to a final concentration of 200 nM. The pseudocolor montages show that the overall level of PI3K signaling is maintained after drug treatment, whereas membrane protrusion is effectively blocked. In some cases, overall cell morphology was largely maintained after drug treatment, whereas in other cases, morphological extensions were lost (compare the top and bottom montages). Correlation coefficients (zero time lag) were calculated for each cell, and the aggregate values are reported in the bar plot as mean ± 95% confidence interval (n = 14). The correlation between morphological extensions and PI3K signaling is modestly reduced on average after cytochalasin D addition because a fraction of the cells lose their extensions with time after treatment (as shown for the cell in the bottom montage). As expected, the correlation between protrusion and PI3K signaling is reduced more significantly after cytochalasin D treatment. Bars, 20 μm.

2.2.5. Branch-and-Pivot Steering Allows Cells to Align with a Chemotactic Gradient

We have shown that PI3K-dependent branching and pivoting of protrusions mediates fibroblast turning behavior and thus governs directional persistence of random migration. Fibroblast migration is directed by chemotactic gradients of platelet-derived growth factor
(PDGF), as during the proliferative phase of wound healing, and therefore we reasoned that cell turning would be important for the cell to become better oriented in the direction of the external gradient and to adjust to transient or competing spatial cues. Under optimal gradient conditions, PDGF elicits robust polarization of PI3K signaling (Schneider and Haugh, 2005), and the pattern of PI3K hot spot localization is quantitatively correlated with the fidelity of PDGF-stimulated chemotaxis (Melvin et al., 2011).
Figure 2.13. Fibroblast reorientation is biased by a PDGF gradient. (a-d) GFP-AktPH-expressing cells were monitored by TIRF microscopy during migration in the presence of alginate microspheres loaded with PDGF. (a) Pseudo-color montage indicating the calculated PDGF concentration field and outline of a chemotactic cell (scale bar = 50 μm). (b) Spatiotemporal maps of protrusion/retraction velocity and PI3K signaling localization for the cell depicted in a. The angular position of maximum PDGF concentration as a function of time is indicated by circle symbols to show the alignment of the cell with the external gradient. The results are representative of 25 cells that exhibited reorientation behavior.
We observed chemotaxis of GFP-AktPH-expressing fibroblasts in the presence of PDGF-loaded, alginate microspheres. By this method, steep chemotactic gradients are achieved, and one can find various arrangements of chemoattractant sources (Melvin et al., 2011). When faced with a choice between two PDGF sources of similar strength, we observe that fibroblasts are sometimes attracted towards both; frequently, the cells choose one or the other, but in this case the steepest PDGF gradient lies between the two sources (Fig. 2.13). To execute the ~90° turn that is required, one end of the cell branches and pivots and maintains strong PI3K signaling in the branch that ultimately aligns towards the sharpest gradient (Fig. 2.13b). The other branch pivots around to the rear and later retracts. In the cohort of chemotaxing cells observed, a total of 30 successful branches were identified and scored according to whether or not one of the branches exhibited markedly higher protrusion velocity or PI3K signaling. The most common outcome, seen 40% of the time, was for both protrusion and signaling to be greater in the branch that became better aligned with the PDGF gradient (Fig. 2.14a). Most often, lamellipodial pivoting resulted in improved alignment of migration directionality, as judged by the change in cell movement angle relative to the gradient (Fig. 2.14b).
Figure 2.14. (a). A total of 30 branching events in the chemotaxing cells were scored according to whether they exhibited biases (markedly more in one branch than the other) in protrusion velocity and/or PI3K signaling localization and, if so, whether the dominant branch experienced the higher or lower PDGF concentration. (b) When the absolute angles of cell movement relative to the gradient (0-180°, with 0° corresponding to perfect alignment) before and after a successful branching event could be clearly determined, these angles were compared by scatter plot. Points below the $y = x$ (dotted) line represent branching events that resulted in improved alignment. (c) Overlaid outlines of GFP-AktPH-expressing fibroblasts, each responding to a PDGF gradient introduced by micropipette (position indicated by an asterisk) oriented roughly perpendicular to the cell’s long axis. The cell on the right shows the more characteristic behavior of cells co-expressing dominant-negative PI3K regulatory subunit. Times after initiation of the gradient are indicated, and scale bars = 20 μm.
In a separate set of experiments, we challenged GFP-AktPH-expressing cells with a PDGF gradient, introduced by flow from a micropipette, oriented such that the gradient was initially oriented at roughly a 90° angle relative to the cell’s long axis. In these experiments, when the cell properly reoriented towards the gradient (which was observed in 6 of 8 motile cells), it invariably did so by successful propagation of the branched state (Fig. 2.14c). In this experimental context, we also tested cells in which recruitment of PI3K was inhibited by co-expression of dominant-negative p85. In these cells, reorientation was achieved less frequently (in 4 of 9 motile cells); more often, these cells continued to move perpendicular to the gradient (Fig. 2.14c). In those instances when the dominant-negative p85-expressing cells reoriented, hot spots of PI3K signaling were observed in the branched protrusions. This suggests that PDGF gradient stimulation induced local recruitment of PI3K (overcoming the antagonism of the dominant negative) or/and local reduction of 3’ phosphoinositide dephosphorylation; evidence for the latter effect in PDGF-stimulated fibroblasts has been reported (Schneider et al., 2005).

Taken together, our results indicate that branch-and-pivot shape changes are initiated stochastically to affect fibroblast reorientation, and that chemotactic gradients bias this process to align cell movement towards an attractant source.

2.3. Discussion

2.3.1. A Conceptual Model of Branch-and-Pivot Turning

Based on our observations and analyses, we propose the following scheme for fibroblast reorientation (Fig. 2.15). First, a lamellipodium develops a newly initiated branch.
This occurs stochastically but with reasonable frequency (once or twice per hour) and is PI3K-independent. In tandem with the newly formed protrusion, PI3K is recruited, and its lipid products accumulate with a characteristic time scale of ~ 1 minute (Schneider and Haugh, 2004). PI3K signaling is required for dilation and stabilization of the branched state; in its absence, the nascent protrusion stalls and eventually retracts. Even after dilating successfully, reorientation is most often unsuccessful. This, we speculate, is linked to the inherently dynamic pattern of PI3K localization, in which distant regions of PI3K signaling globally compete with one another (Weiger et al., 2010). To the extent that PI3K signaling can be maintained, the branched state propagates. We consider this process to be metastable, as it is self-limiting; taken to its fullest extent, the two branches end up at opposite ends of the cell, and the cell executes a near-90° turn. The process is resolved once one of the ends switches from net protrusion to net retraction, at which point the cell is stably polarized and reoriented.
Cells with a defined front and rear at opposite ends are considered stable, and deviations from this morphology are unstable. Nascent branches are initiated stochastically and in a PI3K-independent manner. Following the onset of protrusion, PI3K is localized there; PI3K signaling is required for lateral spreading and stabilization of the new lamellipodium. If PI3K signaling is not interrupted (for too long) in either of the two branches, the branched state is metastable and propagates. A turn of up to 90° is completed once one of the branches stops protruding and reverts to net retraction. The inset outlines a hypothetical feedback loop in which protrusion and PI3K signaling reinforce one another.

2.3.2. On the Emergence and Stabilization of Protrusion Branching

The spatiotemporal protrusion maps (as in Figure 2.1) show that protrusion and retraction are limited to a few extended structures of the cell, providing the basis for fibroblasts’ directional persistence. Protrusion rarely occurs along the cell sides, where
mature actomyosin stress fibers are under contractile tension (although liberation of PA-Rac can apparently overcome this barrier, after a delay). Within the permissive regions, protrusions emerge as discrete bursts, seen as punctate spots on the spatiotemporal maps. The branched structure is favored because new protrusion bursts are forced to emerge in the vicinity of previous ones.

The localized nature of productive protrusion is consistent with the overlapping dynamics associated with the lamellar versus lamellipodial actin networks characterized in epithelial cells (Danuser, 2005; Ponti et al., 2004). The dendritic, lamellipodial network is not required for protrusion per se; rather, through the actions of Arp2/3 and cofillin, it appears to maintain and broaden the leading edge after a localized protrusion event (Gupton et al., 2005). Such a mechanism implies a role for PI3K signaling, which alongside Rac promotes Arp2/3 function at the leading edge by activating WAVE complexes (Lebensohn and Kirschner, 2009; Oikawa et al., 2004; Sossey-Alaoui et al., 2005) (Fig. 2.15, inset). Two observations are consistent with this idea. First, fibroblasts with PI3K inhibited adopt an elongated morphology, with a narrow leading edge but no gross defect in cell speed, similar to those with Rac depleted (Pankov et al., 2005). Second, our results establish that PI3K signaling, like Rac and Cdc42 activation (Machacek et al., 2009; Tsukada et al., 2008), lags the onset of protrusion.

The mechanism outlined above explains how bifurcation of a protrusion might arise, but it does not address the metastability and propagation of the branched state. In that regard, we speculate that myosin-driven maturation of adhesions and stress fibers (Cirit et al., 2010; Vicente-Manzanares et al., 2007) plays a critical role in stabilizing the cleft.
2.3.3. On the Dynamic Coordination of Protrusion and PI3K Signaling

Our spatiotemporal mapping analysis and PA-Rac experiments suggest that PI3K signaling responds to leading-edge protrusion. This could be mediated by, for example, newly formed nascent adhesions or through positive feedback associated with WAVE activation (Millius et al., 2009; Sossey-Alaoui et al., 2005). When protrusion was blocked by cytochalasin D treatment, we observed that PI3K signaling persists but is less dynamic. Therefore, just as PI3K is not required for protrusion but affects its character, protrusion is not required for maintenance of the overall PI3K signaling level but affects its dynamic redistribution under global competition. This “passive” form of positive feedback is consistent with the reported response to local release of dominant-negative Rac: rather than simply inhibiting protrusion in that region, protrusion was induced in distal regions of the cell (Wu et al., 2009).

These conclusions differ somewhat from those of Yoo et al., who studied the localization and function of PI3K signaling in migrating neutrophils, imaged in live zebrafish (Yoo et al., 2010). As in our system, PA-Rac induced protrusion and localization of PI3K signaling in these cells; however, PA-Rac did not elicit migration in neutrophils treated with PI3K inhibitors. This discrepancy might be attributed to differences in cellular/microenvironmental context.

2.3.4. On the Control of Gradient Sensing and Chemotaxis

In their analysis of *D. discoideum* motility, Andrew and Insall noted that protrusion branching is prominent in a variety of cell types, including fibroblasts (Andrew and Insall,
Our analysis reveals a mode of chemotaxis in fibroblasts that is, on the surface, reminiscent of *D. discoideum* motility, in the sense that one of the two branches is favored based on the orientation of the chemoattractant gradient. Just as the mechanics of amoeboid and mesenchymal migration are quite different, so too are the features of the branching phenomena in the two cell types. At least under certain conditions, *D. discoideum* cells branch pseudopods at a regular frequency to execute both modest turns or, through ordered branching, persistent migration (Andrew and Insall, 2007; Bosgraaf and Van Haastert, 2009a; Bosgraaf and Van Haastert, 2009b). In contrast, protrusion branching in fibroblasts occurs stochastically and, if propagated to the bipolar state, yields turns of up to 90°; persistent fibroblast migration is achieved when branching does not occur. In addition to the functional differences, the timing and role of PI3K signaling localization are also distinct. In *D. discoideum*, it has been reported that PI3K signaling patches coalesce prior to pseudopod formation (Postma et al., 2003), and PI3K inhibition reduces the frequency of pseudopod generation (Andrew and Insall, 2007). In fibroblasts, we have shown that PI3K signaling is localized after the onset of protrusion, and accordingly PI3K inhibition does not affect the initiation of branches but rather their ability to propagate.

The branch-and-pivot mechanism mediates large-scale reorientation of chemotaxing cells and, to the extent that the branches are chemoattractant-sensing elements, would aid in gradient perception by extending the branches apart from another. This is not to say that branching is required for gradient sensing or chemotaxis, especially in cells with much broader lamellipodia. On the contrary, once fibroblasts are polarized and migrating with only modest deviations from the gradient axis, they track the gradient by making only small
turns associated with subtle morphology changes (Arrieumerlou and Meyer, 2005; Melvin et al., 2011).

2.4. Materials and Methods

2.4.1. Cell Culture, DNA constructs, and Other Reagents

NIH 3T3 cells (American Type Culture Collection, Rockville, MD) were cultured, and stable expression of GFP- or mCherry-AktPH was achieved by retroviral infection and puromycin selection, as previously described (Weiger et al., 2009). The mCherry-AktPH-pBM-IRES-Puro retroviral vector was constructed by cloning mCherry into the same position as EGFP in the previously described EGFP-AktPH-pBM-IRES-Puro vector (Weiger et al., 2009). Transient co-expression of other constructs was achieved by lipofection. GFP-AktPH-C1, mCherry-AktPH-C1, and dominant-negative p85-α vectors were used as previously (Weiger et al., 2009). The tdTomato-Lifeact plasmid was a gift from James Bear, UNC-Chapel Hill. The GFP-paxillin vector was constructed in the laboratory of Rick Horwitz, University of Virginia (Laukaitis et al., 2001) (Addgene plasmid 15233). mCerulean-PA-Rac (N-term2) construct was a gift from Yi Wu and Klaus Hahn, UNC-Chapel Hill. Human plasma fibronectin was obtained from BD Biosciences (San Jose, CA) and Invitrogen (Carlsbad, CA). PI3K α inhibitor IV was purchased from EMD Biosciences/Calbiochem (San Diego, CA), and cytochalasin D was from Sigma-Aldrich (St. Louis, MO).
2.4.2. Cell Migration Experiments

Glass cover slips were cleaned, sterilized, coated with 10 μg/mL fibronectin (BD Biosciences, San Jose, CA) for 1 h at 37°C, washed with deionized, sterile water, and dried within 30 min of the experiment. Cells were detached with a brief trypsin-EDTA treatment and suspended in the imaging buffer (20 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, 1% v/v fetal bovine serum, 2 mg/mL fatty-acid-free bovine serum albumin). After centrifugation at 100 × g for 3 min, the cells were resuspended in imaging buffer and plated on the fibronectin-coated coverslips at a density of approximately 10,000 cells/mL and allowed to spread for 1-2 hours before imaging. Mineral oil was layered on top of the buffer to prevent evaporation. Chemotaxis experiments using alginate microspheres (a gift from Darrell Irvine, MIT) were performed as described in detail previously (Melvin et al., 2011). PDGF gradients were produced using a micropipette, pulled to a diameter of approximately 30 μm, backfilled with a solution of 2 nM PDGF in imaging buffer, and controlled using a syringe pump (World Precision Instruments) and micromanipulator (Siskiyou Corporation).

2.4.3. Microscopy

Our prism-based TIRF microscope was described in detail previously (Schneider and Haugh, 2005). Excitation of EGFP (488 nm), mCherry (561 nm), tdTomato (561 nm), or teal fluorescent protein (442 nm) was achieved with incident beam energy of roughly 20-50 mJ per image. Water dipping objectives (20X, 0.5 NA and 40X, 0.8 NA Achroplan from Zeiss, Oberkochen, Germany) were used along with a 0.63X camera mount. Digital images were
acquired using a Hamamatsu ORCA ER cooled CCD (Hamamatsu, Bridgewater, NJ) and Metamorph software (Universal Imaging, West Chester, PA).

Photo-activation of PA-Rac was achieved using a 50 W mercury arc lamp passed through a 436/20 nm excitation filter. A focal pattern of light was created by focusing the lamp lens and blocking diffuse light in the light path. A fluorescent dextran solution was used to quantify the spatial profile of excitation, and a threshold was applied to define the region of photo-activation.

2.4.4. Image Analysis

Methods for identification and spatiotemporal mapping of protruded/retracted areas, PI3K signaling hot spots, and extended morphological structures are described below and illustrated in Figure 2.16 (see also sections 4.2.1, 4.2.2, 4.2.5, and 4.2.9). The protruded areas for each time interval are identified as pixels associated with the cell in the present image but not in the previous image, and vice-versa for the retracted areas. For each protruded or retracted pixel, the angle between the pixel and the cell centroid (relative to the negative x-axis) was calculated and rounded to the nearest whole angle. Protrusion or retraction velocity was calculated as the net change in number of protruded/retracted pixels along the indicated angle (multiplied by the pixel size) divided by the change in time. This approach is simple and unambiguous in its implementation, and we find it to be a robust method for image stacks with modest spatial and temporal resolution, as was the case here. More sophisticated protrusion mapping methods have been described (Bosgraaf et al., 2009; Machacek and Danuser, 2006).
Figure 2.16. Identification and spatiotemporal mapping of protruded/retracted areas, PI3K signaling hot spots, and extended morphological structures. (a) Protruded/retracted pixels were mapped according to the whole angle of each pixel relative to the cell centroid, proceeding in a clockwise fashion from the negative x-axis. The lower panel shows the sum of the total protruded or retracted distance along each angle within a single time step, corresponding to protruded and retracted pixels shown in the upper panel in red and blue, respectively. Spatiotemporal maps were constructed by compiling the angle/velocity relationship for each time interval of a time-lapse experiment. (b) PI3K signaling hotspots (shown in red, upper panel) were mapped by summing the background-subtracted PI3K signaling intensity for each segmented pixel lying along a whole angle path between the cell centroid and the hotspot pixel, using the same directional convention as in a. (c) Extended morphological structures (shown in red, upper panel) were identified as local regions of a cell for which the perimeter pixels (black circles, lower panel) are further from the cell centroid than the local average distance from the cell centroid (red curve, lower panel). The spatiotemporal maps of extended cellular structures were created by summing the number of pixels within an extended structure using the same directional convention as for protrusion/retraction and signaling maps.
Image segmentation to identify pixels associated with PI3K signaling hot spots was performed as described in detail previously (Weiger et al., 2010; Weiger et al., 2009). Those pixels were mapped according to their angles relative to the cell centroid, with the value given in the heat map calculated as the sum of background-subtracted fluorescence intensities for all pixels that lie along the indicated angle.

Extended morphological structures were identified as follows. Each fluorescence intensity image was thresholded, and the pixels defining the cell perimeter were indexed according to their relative positions. The local average distance of the cell periphery from the cell centroid (averaged over 20 adjacent pixels) was calculated for each indexed location, and pixels that were greater than 1 \( \mu m \) beyond the local average were considered associated with extended morphological structures. These structures were smoothed by a standard morphological opening operation, and finally the contour of the region was enlarged (dilated) by 5 pixels on each side. Pixels associated with the structures thusly identified were mapped according to their angles relative to the cell centroid, with the value given in the heat map calculated as the number of pixels lying along the indicated angle.

For the purposes of graphical presentation and correlation analysis, the protrusion velocity, hot spot signaling, and morphological extension metrics were smoothed using a weighted linear least squares and a first degree polynomial model using a span equivalent to 5\% of the total spatial and temporal ranges. For the correlation of time derivatives, a span equivalent to 10\% of the total spatial and temporal ranges was used. Cross-correlations between the mapped protrusion, signaling, and morphology metrics, binned into 10-degree angle intervals, were calculated using the Matlab function \texttt{normxcorr2}. To confirm that the
correlations involving local protrusion are not influenced by potential artifacts associated with binning protruded pixels by angle relative to the centroid, the correlation calculations were repeated using a more selective protrusion mapping method. In the modified algorithm, among the protruded or retracted pixels found in a particular angular bin, only those belonging to the contiguous region located furthest from the centroid were included. We confirmed that the use of this approach did not affect any of our conclusions, including the temporal offset between protrusion and signaling.

Cell motility metrics were calculated by manual thresholding of the TIRF images to identify the cell contact area. For each cell, cell speed was calculated as the mean of the instantaneous displacement of the contact area centroid, sampled every 12 minutes. Migration path D/T was calculated by dividing the overall displacement of the cell centroid by the sum of the distances moved along the path of the centroid, sampled every 12 minutes. The protruded area was calculated as the mean value of the instantaneous protruded area, sampled every 12 minutes. The cell path axis ratio was calculated as the ratio of the minor and major axes of an ellipse having the same normalized second central moments as the cell path, which was determined by creating a pileup of the cell contact areas taken at 2-minute intervals (Fig. 2.17).
Figure 2.17. Determination of cell path axis ratio. A cell track is defined as the area obtained by overlaying the cell’s contact area in each frame, akin to what is measured in a phagokinetic track assay. The best fit of each cell track to an ellipse is determined using MATLAB, and the associated cell path axis ratio is calculated by dividing the length of the minor axis (green lines) by that of the major axis (red lines). Representative tracks of control and dominant-negative p85-expressing cells are shown. Scale bars = 30 μm.

2.5. Conclusions & Outlook

2.5.1 Conclusions

Previously we had correlated PI3K activity with the direction of cell migration. Here, we have identified specific function for PI3K in mesenchymal migration which is consistent with our previous results. Randomly migrating fibroblasts reorient polarity through PI3K-dependent branching and pivoting of protrusions. This mechanism of mesenchymal cell-turning is revealed through analysis of spatiotemporal protrusion/retraction maps. PI3K signaling follows a similar pattern and is typically localized to extensions. PI3K inhibition does not affect the initiation of newly branched protrusions, nor does it prevent protrusion
induced by photo-activation of Rac. Rather, PI3K signaling increases after, not before, the onset of local protrusion and is required for the lateral spreading and stabilization of nascent branches. Inhibition of PI3K reduces protruded areas and modestly increases cell speed. Peak PI3K activation is protrusion dependent as shown by protrusion induced by photo-activation of Rac in the absence and presence of actin polymerization inhibitors. During chemotaxis, the branch experiencing the higher chemoattractant concentration is favored, and thus the cell reorients so as to align with the external gradient.

3.5.2 Outlook

While significant progress has been made in the study to determine the specific role of PI3K in migration more is needed to improve our understanding of several areas; primarily, as to what dictates the formation and orientation of new branches and how PI3K signaling is localized to them. More work is still needed to determine if PI3K is explicitly required for chemotaxis as well as other directed migration cues, such as in the context of haptotaxis and durotaxis. Many of molecular details of PI3K activation on the onset of protrusion still need to be elucidated. The pathway of positive feedback through adhesions has long been suggested but is poorly understood and the specific molecular requirements are unknown. While the timing of peak PI3K signaling and signaling increases relative to protrusion was investigated, higher temporal and spacial resolution imaging is required to determine the precise location and timing of origins of PI3K signaling. Several of these points are investigated in detail in the next chapter.
2.6. Acknowledgements

We thank Y. Wu and K. Hahn and J. Bear (University of North Carolina-Chapel Hill) and Darrell Irvine (MIT) for reagents. This work was supported by NSF grant CBET-0828936, and E.S.W. was supported by the Cell Migration Consortium under National Institutes of Health grant U54-GM064346.

2.7. References


CHAPTER 3  

F-actin Bundles Direct the Initiation and Orientation of Lamellipodia Through Adhesion-Based Signaling

*Adapted from Johnson HE, King SJ, Asokan SB, Rotty JD, Bear JE, and Haugh JM. F-actin bundles direct the initiation and orientation of lamellipodia through adhesion-based signaling. Journal of Cell Biology, Accepted.
3.1. Introduction

The importance of cell migration in development, immunity, wound repair, and cancer progression, has long been appreciated. Unifying these various physiological and pathological contexts is a common design principle: the ability of migrating cells to change or maintain directionality as they monitor their microenvironment for spatial cues (Bear and Haugh, 2014; Petrie et al., 2009). However, different cell types use fundamentally distinct mechanisms to achieve this objective. Whereas amoeboid cells such as leukocytes exhibit a robustly polarized and excitable cytoskeleton, which only needs to be subtly perturbed by soluble cues to bias cell movement (i.e., in chemotaxis) (Arrieumerlou and Meyer, 2005; Iglesias and Devreotes, 2012; Xu et al., 2003), mesenchymal cells such as fibroblasts exhibit weakly polarized migration phenotypes and respond to both chemical and physical cues (Bear and Haugh, 2014; Lara Rodriguez and Schneider, 2013). The latter include spatial gradients of immobilized, adhesive ligand density (haptotaxis) and of mechanical stiffness (durotaxis).

The most prominent cytoskeletal structure that drives cell locomotion is the lamellipod, a broad, fan-shaped protrusion with an F-actin-rich leading edge. The dendritic architecture of the leading-edge F-actin array is formed by integration of the Arp2/3 complex, which nucleates assembly of new actin filaments from existing ones and thus largely controls the rate of actin polymerization that drives lamellipodial protrusion (Rotty et al., 2013). This activity is in turn controlled by a host of signaling molecules, most notably the small GTPase Rac and the phospholipid phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which cooperate to activate the SCAR/WAVE regulatory complex upstream of
Arp2/3 (Lebensohn and Kirschner, 2009). PIP3 is produced by type I phosphoinositide 3-kinases (PI3Ks) and, like GTP-bound Rac, is focally enriched in protruding lamellipodia (Kraynov et al., 2000; Weiger et al., 2009). The weakly polarized morphology of fibroblasts is typically characterized by multiple lamellipodia, which exhibit intermittent protrusion and signaling and compete with one another to determine the overall direction of migration (Petrie et al., 2009; Weiger et al., 2010).

Previously, we characterized a mechanism by which fibroblasts execute large-scale changes in orientation by extension of nascent lamellipodia, which most often form by bifurcation of the dominant lamellipod; if the two branches successfully propagate to their fullest extent, a 90-degree turn is achieved (Welf et al., 2012). Our experiments revealed a specific role for PI3K signaling in lamellipodial spreading, which is required to maintain the propagation of the branches, whereas initiation of branching is PI3K-independent. Accordingly, we found that increases in local PI3K signaling lag behind the acceleration of protrusion. These findings established the macroscopic morphodynamics of fibroblast migration that allow efficient reorientation of directionality, e.g., in response to external cues, but they also spurred a new set of questions aimed at the subcellular level. How do newly branched lamellipodia form? What determines the distinct directions of lamellipodial extension? Here, we show that F-actin bundles containing fascin-1, which often manifest as filopodia, seed the formation and set the orientations of nascent lamellipodia.

Filopodia are narrow, dynamic, finger-like protrusions with established roles in neuronal communication and development (Teddy and Kulesa, 2004), epithelial cell-cell adhesion (Vasioukhin et al., 2000; Wood et al., 2002), and cell motility; yet, the precise
contexts in which filopodia affect cell migration are largely unknown. The concept that filopodia generally serve as sensing organelles has been broadly speculated (Mattila and Lappalainen, 2008; Ridley et al., 2003), yet there is scant evidence supporting this notion except in the neuronal context (Davenport et al., 1993; Dent et al., 2011). In fibroblasts, it has been observed that filopodia seed the formation of distinct lamellipodia during the transition from isotropic to anisotropic spreading on an adhesive surface (Guillou et al., 2008), but the functional and mechanistic connections to random or directed locomotion are not yet established.

Critical to the assembly and stability of filopodia is the crosslinking of parallel actin filaments. Fascin is one of the major actin-bundling proteins in filopodia, though it has been shown to promote filopodia independent of its crosslinking function (Zanet et al., 2012). Among the three isoforms of fascin, only fascin-1 is broadly expressed in mesenchymal cells. Depletion of fascin-1 or impairment of fascin-actin binding significantly reduces the number of filopodia, whereas the constitutively active (S39A) actin-binding mutant increases the number and average length of filopodia (Li et al., 2010; Vignjevic et al., 2006).

In this work, we employed high-resolution imaging and analysis of subcellular dynamics, combined with directed migration assays, to elucidate a key function of F-actin bundles/filopodia in orchestrating lamellipodia during both random and directed migration of mesenchymal cells. Our results implicate both structural and signaling roles of fascin-containing bundles in this process and thus connect the subcellular and macroscopic morphodynamics of cell locomotion.
3.2. Results

3.2.1. Filopodia Direct Formation of Newly Branched Lamellipodia

To study the formation of nascent lamellipodia, high-resolution total internal reflection fluorescence (TIRF) movies of cells randomly migrating on fibronectin were acquired and analyzed. Owing to the exquisite z-resolution of TIRF illumination, adherent filopodia/microspikes of migrating NIH 3T3 fibroblasts were readily visible. We found that such filopodia typically preceded the emergence and oriented the protrusion of new lamellipodia, which most often branch off from existing ones (Figure 3.1). Accordingly, spatiotemporal maps of protrusion and retraction activity show that large-scale branching of lamellipodia coincides with protrusion over filopodia (Fig. 3.1a). A method for identifying and tracking filopodia was devised (see section 4.2.6), allowing a clearer visualization of these structures in relation to cell shape dynamics. Lamellipodia were also identified automatically, based on a combination of morphological and protrusion criteria, and spatiotemporal overlap of lamellipodia and filopodia was assessed. Of the lamellipodia identified (n = 1005, in 21 cells analyzed), 72% overlapped with filopodia; 55% of the lamellipodia overlapped with pre-existing filopodia (Fig. 3.1b).
Figure 3.1. (a) Pseudocolored TIRF images of a NIH 3T3 cell expressing the EGFP-AktPH biosensor, representative of 28 cells in 4 independent experiments. The montage shows that filopodia template nascent lamellipodia, which typically branch from existing ones during random migration on fibronectin (left). Scale bar = 10 μm. The corresponding spatiotemporal map of protrusion/retraction velocity, overlaid on regions of morphological extension (gray; see Methods) (center) indicates the timing and angular locations of these branching events relative to the cell centroid (clockwise from the negative x-axis). The colored arrows on both the montage and map show these events. The accompanying lamellipod/filopod overlap map (right) indicates pixels associated with putative lamellipodia (green), filopodia (orange), or overlap of the two (black). (b) Analysis of lamellipod/filopod overlap. Indicated are the percentages of lamellipodial regions that emerged overlapping a pre-existing filopod, emerged without a pre-existing filopod but overlapped with at least one filopod thereafter, or never overlapped with a filopod.
We next asked whether or not the tendency to initiate protrusion around filopodia is a general property of lamellipodia. To test this, we used photoactivatable Rac (Wu et al., 2009) to locally induce Rac and Arp2/3 activation in NIH 3T3 cells. Although the spots of photoactivation were aimed near and not centered over visibly adhered filopodia, the ensuing protrusions occurred preferentially along those filopodia (Fig. 3.2a). This protrusion bias was seen 92% of the time (66/72), and PI3K signaling was enriched along the bundle in all but one of those instances. In a related experiment reported previously (Wu et al., 2012), Arp2/3-depleted fibroblasts were microinjected with purified Arp2/3 complex to induce lamellipodial protrusion globally. Analysis of this sequence confirms that the protrusions originated along filopodia before coalescing to form broad lamellipodia (Fig. 3.2b).
Figure 3.2. (a) TIRF montage, representative of 67 cells showing that local photoactivation of Rac (red spot) in NIH 3T3 cells induces protrusion and PI3K signaling preferentially over actin bundles (red arrows). Frames labeled with red circles show the cell immediately before activation of Rac at the indicated position. The other frames show the cell right before activation was ceased at that location and allowed to recover until the next ‘activation start’. Scale bar = 20 μm. (b) Montage showing a time course following microinjection of Arp2/3 complex into Arp2/3-depleted IA32 MEFs (Wu et al., 2012). Red arrows indicate filopodia where protrusion initiated. Scale bar = 20 μm.

3.2.2. Expression of the Actin-Bundling Protein, Fascin-1, Tunes Cell Morphology

To monitor filopod/lamellipod dynamics in better detail, we employed fluorescent protein (FP)-tagged fascin-1 as a live-cell marker of F-actin bundles. In FP-fascin-expressing fibroblasts, we observed the same templating of branched protrusions as before, but here we were not limited by the appearance of filopodia and could readily see F-actin bundles embedded behind the leading edge (Fig. 3.3a and Fig. 3.4a). The protrusions that emerge around bundles are labeled by FP-Lifeact, indicating F-actin network at the leading edge, characteristic of lamellipodia (Fig. 3.4b). Analysis of lamellipodial overlap with F-actin bundles and filopodia in FP-fascin-expressing fibroblasts showed that 84% of the
lamellipodia (n = 1429, from 31 cells) overlapped with pre-existing F-actin bundles or filopodia (Fig. 3.3b). Conversely, most F-actin bundles/filopodia never overlap with lamellipodia during their lifetime (Fig. 3.4c), indicating that emergence of lamellipodia is rare compared with the appearance and disappearance of adherent filopodia.
Figure 3.3. (a) TIRF montage (inverted grayscale) of a branching event in a NIH 3T3 cell expressing EGFP-fascin to mark F-actin bundles (left), representative of 31 cells in 13 independent experiments (see Figure 3.4a for another example). Scale bar = 10 μm. The corresponding protrusion/retraction and lamellipod/filopod overlap maps are shown at right. (b) Lamellipod/filopod overlap analysis as shown in Fig 3.1b; here, both putative filopodia and submembranous bundles labeled by FP-fascin were included in the analysis.
**Figure 3.4.** (a) Left: TIRF montage of an NIH 3T3 fibroblast expressing EGFP-fascin (inverted grayscale), with prominent F-actin bundles marked by red arrows. Scale bar = 10 μm. Right: Corresponding spatiotemporal map of protrusion and retraction. The initiation of lamellipodial branching apparent in the latter two panels of the montage at left manifests on the map as an upside-down ‘U’ shape (roughly, between 115 and 140 degrees relative to the centroid starting at ~ 8 minutes in this time course). (b) TIRF montage of an NIH 3T3 co-expressing tdTomato-Lifeact and EGFP-fascin, illustrating F-actin organization in nascent lamellipodia and bundles. (c) Analysis of the percentages of filopodia that (from top to bottom): appear before an overlapping lamellipod; appear after, and persistent after, an overlapping lamellipod; appear after, and disappear during the lifetime of, an overlapping lamellipod; or appear and disappear with no overlap with lamellipodia. (d) Quantification of cell speed, by cell centroid tracking for control (n = 21) and fascin-overexpressing (OE) (n = 31) cells. The data are presented as mean ± 95% confidence interval.
Figure a shows time-lapse images of cell behavior with red arrowheads indicating changes over time.

Figure b presents images of Td-Tom-Lifeact and EGFP-Fascin staining at different time points (0 min, 10.5 min, 7.5 min, and 12 min).

Figure c displays a table with Akt-PH Fascin values for different conditions.

Figure d illustrates the comparison of cell speed between control and Fascin OE conditions.
Based on the apparent role of F-actin bundles in cell morphodynamics, and considering that fascin-1 promotes the stability of filopodia, we hypothesized that cell shape is sensitive to fascin-1 expression level. To quantify cell morphology, we determined the number of extended structures (e.g., lamellipodia) in single images of many cells. When fascin-1 was overexpressed in NIH 3T3 fibroblasts, the mean number of morphological extensions was significantly increased relative to control cells, consistent with indications in other cell types (Yamashiro et al., 1998). The incidence of cells with six or more extensions was dramatically increased, along with reduced incidence of cells exhibiting three or fewer extensions, in the fascin-overexpressing population (Fig. 3.5a); in contrast with cell morphology, overall migration speed was not significantly altered by fascin overexpression (Fig. 3.4d). Conversely, depletion of fascin-1 by short hairpin RNA (shRNA) interference had the opposite effect on cell morphology. Expression of two shRNAs, which yielded ~90% knockdown of fascin-1 each and > 97% knockdown when combined, progressively reduced the mean number of morphological extensions (Fig. 3.5b). To confirm the specificity of this approach, expression of human fascin-1 (which does not contain the sequences targeted in murine fascin-1) in the double-knockdown background fully rescued the normal phenotype (Fig. 3.5b).
Figure 3.5. (a) Representative TIRF images of NIH 3T3 cells expressing EGFP-AktPH or co-expressing mCherry AktPH and EGFP-fascin are shown. Scale bar = 20 μm. The bar graph shows the mean numbers of morphological extensions for the control (blue, n = 152) and fascin-overexpressing (red, n = 113) cells; error bars show 95% confidence intervals. The histogram shows the corresponding distributions. (b) NIH 3T3 cells were depleted of fascin-1 by two targeting shRNAs, expressed separately or in combination. The immunoblot confirms shRNA-mediated loss of fascin-1 expression levels relative to a non-targeting control shRNA, with GAP-DH as a loading control. Representative TIRF images of these cells as well as of cells in which fascin-1 expression was rescued are shown. Scale bar = 20 μm. The bar graph shows the mean number of extensions quantified for each population of cells (n ≥ 140 for each condition); error bars show 95% confidence intervals.
3.2.3. PI3K Signaling Colocalizes with F-actin Bundles and is Enriched in Filopodia

Our previous work showed that PI3K signaling locally increases following the onset of protrusion, stabilizing nascent lamellipodia to affect macroscopic changes in cell morphology (Welf et al., 2012). With the present insight that F-actin bundles/filopodia initiate and orient this process, we asked whether or not these structures also harbor PI3K signaling and thus serve as primers for actin polymerization. Consistent with this idea, in NIH 3T3 fibroblasts co-expressing FP-fascin and FP-AktPH (a translocation biosensor for 3’ phosphoinositides), the two typically colocalize near the leading edge and especially at the bases of filopodia during the early phase of lamellipod formation, when protrusion accelerates (Fig. 3.6a).

Is PI3K signaling enriched within filopodia as well? This question is not straightforward, given the size of these structures: ~ 100 nm in diameter, below the resolution limit and comparable to the depth of TIRF illumination. Therefore, we assessed enrichment of PI3K signaling in filopodia by ratio imaging. The average TIRF intensity of FP-AktPH domain was normalized by that of a FP cytoplasmic volume marker; this fluorescence ratio, measured for each filopodium, was normalized again by the fluorescence ratio of a region near the center of the contact area (see section 4.2.8 for technical notes). PI3K signaling in filopodia is considered significant if the resulting quantity, the enrichment ratio E, is significantly greater than 1. Averaged over the lifetime of each filopodium (identified by the aforementioned segmentation algorithm; see section 4.2.6), the mean value of E = 3.3, whereas pharmacological inhibition of PI3K yielded a mean value of E = 1.1 (Fig. 3.6b).
Thus, the ratio imaging approach was validated and indicates that filopodia harbor substantial PI3K signaling.

Having devised a method for detecting PI3K signaling in filopodia, we asked whether or not this activity was present prior to the emergence of lamellipodia. In time-lapse movies of 27 cells subjected to ratio imaging, we identified those instances when lamellipodia emerged over filopodia without marked pre-existing enrichment of PI3K signaling in the proximal region of the plasma membrane (i.e., as would be indicated by inspection of the FP-AktPH channel alone). In 78% of those instances (151/194), PI3K signaling was enriched in the filopodial structures beforehand, and was thereafter elevated in the lamellipod (Fig. 3.6c). These findings establish filopodia as both structural guides and signaling hubs that prime the emergence of lamellipodia.
Figure 3.6. (a) Montage of PI3K signaling (pseudocolored) and fascin-1 localization (inverted grayscale) during protrusion over actin bundles in an NIH 3T3 cell, representative of 31 cells viewed. Scale bar = 10 μm. (b) NIH 3T3 cells co-expressing mCherry-AktPH and TFP as a volume marker were used for ratiometric analysis. Average enrichment ratios (mean ± 95% confidence interval) of PI3K signaling in filopodia (left) and the associated histograms (right) for untreated cells (blue, n = 724 filopodia) versus cells treated with 1 μM PI3K-α inhibitor IV (red, n = 119 filopodia) are shown. (c) Montage of mCherry-AktPH/TFP ratio (pseudocolored) showing PI3K signaling enrichment preceding lamellipodial protrusion over an actin bundle (left). Scale bar = 5 μm. For each cell, the percentage of instances in which PI3K signaling first increased in filopodia was determined for each cell (right; n = 27 cells, 194 protrusions total over actin bundles). The red dot indicates mean ± 95% confidence interval across cells.
3.2.4. Nascent Adhesions Form Along F-actin Bundles in Tandem with Propagation of PI3K Signaling

Integrin-mediated adhesion is responsible for attachment of filopodia to ECM (Galbraith et al., 2007; Partridge and Marcantonio, 2006), while in lamellipodia, it is established that adhesion complexes foster activation of PI3K and Rac, leading to actin polymerization mediated by Arp2/3 complex (Chen and Guan, 1994; Cox et al., 2001). To test whether or not adhesions nucleate beneath F-actin bundles with the appropriate timing relative to lamellipod formation, we imaged NIH 3T3 fibroblasts co-expressing FP-fascin and FP-paxillin, a marker of adhesion complexes. Adhesions were found along the length of fascin-containing bundles, with the highest density of paxillin typically at the base (Fig. 3.7a). Most often, such adhesions at the cell periphery lack zyxin, a marker for mature adhesions and stress fibers (Yoshigi et al., 2005; Zaidel-Bar et al., 2003) (Fig. 3.8a&b). Small adhesions often appeared at the tips of filopodia, followed by adhesion growth along the length of the bundle as the nascent lamellipod protruded over it. In cells co-expressing FP-paxillin and FP-AktPH, the large adhesions present at the bases of filopodia colocalize with the emergence of PI3K signaling, during the early phase of lamellipodial protrusion (Fig. 3.7b). This colocalization was observed in 43/49 (88 %) of lamellipodia observed to protrude over filopodia in these cells.
Figure 3.7. (a) Montage of PI3K signaling (pseudocolored) and fascin-1 localization (inverted grayscale) during protrusion over actin bundles in an NIH 3T3 cell, representative of 31 cells viewed. Scale bar = 10 μm. (b) NIH 3T3 cells co-expressing mCherry-AktPH and TFP as a volume marker were used for ratiometric analysis. Average enrichment ratios (mean ± 95% confidence interval) of PI3K signaling in filopodia (left) and the associated histograms (right) for untreated cells (blue, n = 724 filopodia) versus cells treated with 1 μM PI3K-α inhibitor IV (red, n = 119 filopodia) are shown.
Figure 3.8. (a) Two-color TIRF imaging of zyxin and paxillin in NIH 3T3 cells confirms that mature, zyxin-containing adhesions are found some distance behind the leading edge. (b) Two-color TIRF imaging of fascin and zyxin in NIH 3T3 cells confirms that zyxin-containing adhesions are typically located behind F-actin bundles at the periphery. (c) TIRF images of FP-AktPH-expressing NIH 3T3 cells, plated on either fibronectin or poly-lysine, before and after inhibition of Src-family kinases (PP2, 10 µM). The images are representative of 13 cells viewed for fibronectin and 19 cells viewed for poly-lysine.

These colocalization studies suggest that adhesions mediate activation of PI3K signaling and other pathways that promote Arp2/3-based, lamellipodial protrusion around F-actin bundles. To test this hypothesis, we assessed the role of focal adhesion kinase (FAK), a
known intermediate in integrin-mediated activation of PI3K (Berg and Cheney, 2002; Reiske et al., 1999). Pharmacological inhibition of FAK activity, which prevents FAK autophosphorylation of the tyrosine residue that engages type IA PI3Ks, ablated the localization of PI3K signaling to putative lamellipodia (Fig. 3.9). Whereas FAK inhibition significantly reduced PI3K localization in the cells plated on fibronectin as before, this was not the case for cells plated on poly-lysine, which promotes integrin-independent adhesion of cell membranes (Fig. 3.9). Inhibition of the Src family of protein tyrosine kinases, which are recruited to adhesion complexes as well as other signaling complexes (Cuevas et al., 2001; Plopper et al., 1995), also ablated localization of PI3K signaling in cells plated on fibronectin; and this treatment partially reduced PI3K signaling in cells plated on poly-lysine (Fig. 3.8c).
Figure 3.9. TIRF images of FP-AktPH-expressing NIH 3T3 cells, plated on either fibronectin or poly-lysine, before and after FAK inhibition (10 μM FAK inhibitor II). For each cell, the after/before ratio compares the difference between the mean intensity of the morphological extension with the highest intensity and the mean intensity of the center region of the contact area; a value significantly less than 1 indicates that the pattern became more uniform after FAK inhibition. Values are reported as mean ± 95% confidence interval for the fibronectin (n = 19) and poly-lysine (n = 24) data.
3.2.5. Arp2/3-Driven Protrusion Amplifies Adhesion-Based Signaling by Positive Feedback

The observation that photoactivation of Rac induces redistribution of PI3K signaling (Fig. 3.2a), by a mechanism that requires actin polymerization (Welf et al., 2012), suggested that the signaling circuit controlling Arp2/3 activation in fibroblasts operates under positive feedback. This is plausible because lamellipodial protrusion results in formation of nascent adhesions that are in transient contact with the dendritic F-actin network (Vicente-Manzanares et al., 2009). Consistent with such a mechanism, after Arp2/3 was pharmacologically inhibited in NIH 3T3 cells co-expressing FP-fascin and FP-AktPH, most of the numerous lamellipodia retracted, in concert with loss of PI3K signaling (Fig. 3.10a). FP-paxillin labeling confirmed that small adhesions near the leading edge were also lost after Arp2/3 inhibition (Fig. 3.11a), as well as in other contexts where lamellipodia are impaired: after PI3K inhibition and in fascin-depleted cells (Fig. 3.11b&c). The effect of Arp2/3 inhibition on PI3K signaling was not observed in cells plated on poly-lysine (Fig. 3.10b), linking the phenomenon to integrin-mediated adhesion. To rule out off-target effects of the drug on PI3K signaling, we performed similar experiments with a different fibroblast line, in which conditional knockout of the Arpc2 (p34) subunit of the Arp2/3 complex was established (Rotty et al., 2014). Whereas uninduced (wild-type) cells treated with Arp2/3 inhibitor showed the same ablation of PI3K signaling seen in NIH 3T3 cells, cells treated with tamoxifen to induce Cre-based silencing of Arpc2 showed no marked changes in distribution of PI3K signaling upon Arp2/3 inhibition (Fig. 3.10c).
Figure 3.10. (a) TIRF images of a NIH 3T3 cell co-expressing mCherry-AktPH and EGFP-fascin before and after inhibition of Arp2/3 complex by 50 μM CK666 (representative of 15 cells in 6 independent experiments). Scale bar = 20 μm. (b) CK666 treatment (100 μM) does not ablate localized PI3K signaling in FP-AktPH-expressing NIH 3T3 cells plated on poly-lysine (representative of 42 cells). Scale bar = 20 μm. (c) FP-AktPH-transfected fibroblasts with conditional knockout of Arpc2 (p34) were either uninduced (WT) or induced with tamoxifen (Arpc2/-). TIRF images acquired before and after CK666 treatment are shown (representative of 13 cells each). Scale bars = 20 μm.
Figure 3.11. (a) Two-color TIRF imaging of Lifeact and paxillin before and after inhibition of Arp2/3 complex by 100 μM CK666. The arrows show where small adhesions were lost following collapse of lamellipodia. (b) Same as (a) but with inhibition of PI3K (1 μM PI3K-α inhibitor IV). (c) Two-color TIRF imaging of AktPH or Lifeact biosensor with tagged paxillin in cells depleted of fascin-1. (d) Left: Kymograph of protrusion width during periodic protrusion waves as in Figure 3.12. Lines are drawn through the waves and used to calculate the velocity of each wave. Right: Histogram indicating velocities of protrusion dilation waves (n = 48), estimated from kymograph as shown on the left. These were taken from high-resolution videos of 11 recurrent protrusions observed in 6 different cells.
A hallmark of positive feedback is the phenomenon of traveling waves. We observed recurring, traveling waves of protrusion/retraction directed along a subset of filopodia (n = 11, in 6 cells analyzed this way).  (Fig. 3.12).  In all instances, PI3K signaling was most intense at the base of the structure.  We developed an automated analysis to plot the width of protrusions as a kymograph, i.e., as a function of time and position parallel to protrusion. From these kymographs we estimated a mean wave velocity of approximately 3 μm/minute (Fig. 3.11d).

**Figure 3.12.** Protrusion dilation waves as shown by pseudocolored mCherry-Akt-PH (left) representative of 11 such sequences viewed in 6 cells. Scale bar = 10 μm. The protrusion width and FP-AktPH intensity are plotted for the same sequence on kymograph-like plots (right).
3.2.6. Fascin-1 Depletion Blocks Fibroblast Haptotaxis but Not Chemotaxis

Having elucidated the dynamic relationship between fascin-containing F-actin bundles and formation of lamellipodia that affects large-scale changes in cell morphology, we sought to test the functional role of fascin-1 in different modes of directed cell migration. For this we used IA32 mouse fibroblasts, which exhibit robust tactic migration in response to a gradient either soluble PDGF (chemotaxis) or immobilized fibronectin (haptotaxis) established in a microfluidic chamber (Wu et al., 2012). In this cell line, we confirmed the basic findings that fascin-containing bundles serve as templates for formation of lamellipodia and activation of PI3K signaling (Fig. 3.13a). As in NIH 3T3 cells, we depleted fascin-1 expression by shRNA interference and achieved rescue in this background by expression of homologous fascin-1 (Fig. 3.13b). In PDGF chemotaxis assays, fascin-1 depletion yielded a modest reduction in the mean forward migration index (FMI); however, the difference is not statistically significant (Fig. 3.13c). In contrast, fascin-1 depletion ablated haptotaxis, a phenotype that was reversed by rescue of fascin-1 expression (Fig. 3.13d). Under both chemotaxis and haptotaxis conditions, metrics of unbiased cell migration efficiency – mean speed and persistence (D/T ratio) – were not significantly affected by fascin-1 depletion. We conclude that fascin-1 performs an important function in directed migration of fibroblasts, but in a context-dependent manner.
Figure 3.13. (a) TIRF montage, representative of 13 cells, showing filopodia-templated protrusion in IA32 MEFs co-expressing EGFP-fascin (inverted grayscale) and mCherry Akt-PH (pseudocolored). Scale bar = 10 μm. In the example shown, protrusions over filopodia coalesce to form a broad lamellipod. (b) Representative immunoblot showing relative fascin-1 expression levels in the non-targeting shRNA, fascin shRNA (#1), and fascin rescue IA32 cells used in directed migration experiments. (c) Chemotaxis assay results: wind-rose plots showing the distributions of overall cell migration directionality, expressed as an angle relative to an external gradient of PDGF, are shown for control (n = 74) and fascin-1 shRNA (n = 131). Mean FMI, velocity and persistence (D/T) are displayed +/- 95% confidence intervals. (d) Haptotaxis assay results are shown for control (n = 128), fascin-1 shRNA (n = 124), and fascin rescue (n = 57) tracks. Mean FMI, velocity and persistence (D/T) are displayed +/- 95% confidence intervals.
a

EGFP-Fascin

mCh-Akt-PH

0 min

7 min

10.5 min

20.5 min

b

Control shRNA

Fascin

GAP-DH

C

Control shRNA

Fascin shRNA

FMI

0.19 ± 0.08

Speed (µm/hr)

17.4 ± 1.71

D/T Ratio

0.53 ± 0.05

FMI

0.14 ± 0.05

Speed (µm/hr)

15.3 ± 1.82

D/T Ratio

0.48 ± 0.04

d

Control shRNA

Fascin shRNA

Fascin shRNA + Rescue

FMI

0.20 ± 0.05

Speed (µm/hr)

10.5 ± 0.84

D/T Ratio

0.47 ± 0.04

FMI

0.05 ± 0.06

Speed (µm/hr)

10.8 ± 1.28

D/T Ratio

0.52 ± 0.04

FMI

0.25 ± 0.08

Speed (µm/hr)

14.8 ± 1.37

D/T Ratio

0.52 ± 0.04
3.3. Discussion

3.3.1. Cooperation of Bundled and Dendritic F-actin in Formation and Orientation of Lamellipodia

Based on the evidence provided here, together with previous work, we construct the following sequence of processes that link subcellular dynamics to macroscopic changes in cell shape (Fig. 3.14). (1) F-actin bundles form at the periphery of the cell, often manifest as filopodia. Larger adhesions are typically present at the bases of these actin bundles, while smaller adhesions typically form at the tip and eventually along the length of the bundle (Fig. 3.7a), consistent with published studies (Nemethova et al., 2008; Schafer et al., 2010). (2) These structures seed and orient new lamellipodia (Figs. 3.1 – 3.4), characterized by Arp2/3-mediated actin polymerization, and a positive feedback loop involving adhesion formation and a FAK/PI3K signaling pathway sustains the spreading (extension and dilation) of the nascent lamellipod. (3) As it does so, new F-actin bundles/filopodia emerge/adhere (Fig.3.3a), consistent with previous indications that Arp2/3-mediated protrusion promotes bundle formation (Korobova and Svitkina, 2008; Yang and Svitkina, 2011); this spatial relationship explains the apparent branching of lamellipodia that results in large-scale reorientation of overall cell migration direction (Welf et al., 2012). Whether before or after lamellipodial branching, the protrusion(s) can retract, and the F-actin bundles are left behind to template future waves of lamellipodial protrusion.
Figure 3.14. Conceptual model of morphodynamics directed by F-actin bundles. Peripheral actin bundles, e.g. filopodia, template clustering of nascent adhesions and harbor PI3K signaling. The bundles direct lamellipodia, formed by Arp2/3-mediated actin polymerization, by setting the preferred sites and directions of protrusion initiation and propagation, respectively. An adhesion-based positive feedback loop (inset) amplifies PI3K signaling as the lamellipod spreads, promoting additional activation of Arp2/3 complex and formation of new actin bundles. The new bundles can direct initiation of distinct lamellipodia, seemingly branched from existing ones. Propagation of lamellipodia is self-limiting or metastable, and therefore protrusion and PI3K signaling cease, and the lamellipodia at least partially retract but typically leave the F-actin bundles intact. Hence, the cycle can repeat, sometimes as a series of traveling protrusion waves.
3.3.2. Priming of PI3K Signaling by Actin Bundles

Ratiometric TIRF imaging revealed substantial PI3K signaling in adherent filopodia (Fig. 3.6b), following a precedent reported for dendritic filopodia in neurons (Luikart et al., 2008). We detected this signaling activity even before the emergence of nascent lamellipodia (Fig. 3.6c) and the more readily visible increases in PI3K signaling that follow (Welf et al., 2012). As shown in section 4.2.8, the apparent enrichment of the PIP3 biosensor FP-AktPH in filopodia is partially attributed to geometric considerations; it is estimated that the entire membrane sheath of a filopodium is within the field of TIRF illumination. Considering also the uncertainties about partitioning of the cytoplasmic FP-AktPH pool in/out of filopodia and the net diffusion of PIP3 from the filopodial sheath to the adjacent region of the plasma membrane, the present data do not allow accurate quantification of the extent of PI3K activity enrichment in these structures. The possibility that the density of PIP3 is enhanced by reduction of its turnover (Schneider et al., 2005), i.e., by regulation of lipid dephosphorylation, must also be considered. If achieved by specific localization of PI3K, our colocalization, FAK inhibition, and poly-lysine control experiments implicate the adhesion complexes that form underneath adherent F-actin bundles/filopodia as both the structural and signaling linchpins.

Our results further suggest that PI3K and other signaling activities associated with F-actin bundles prime a positive feedback loop in which adhesions and FAK/PI3K signaling both promote and respond to Arp2/3-mediated actin polymerization and membrane protrusion (Fig. 3.10). Excitability and traveling waves are hallmarks of systems with positive feedback and have been characterized in other migrating cell types (Allard and
Mogilner, 2013; Huang et al., 2013). The traveling waves of protrusion and PI3K signaling that we sometimes observe (Fig. 3.12) suggest that there is negative regulation as well. Candidates for such regulation mechanisms include mechanical feedback involving Rho signaling to Myosin II (Guilluy et al., 2011; Welf et al., 2013) and an incoherent feedforward loop wherein active Rac both promotes and inhibits Arp2/3 complex, through WAVE and Arpin respectively (Dang et al., 2013).

3.3.3. The Role of Fascin-Containing Bundles in Directed Cell Migration

Our functional data demonstrate that fascin-1 depletion impairs lamellipodia formation and haptotactic sensing of fibroblasts on ECM. Previous work showed that depletion of Arp2/3 complex ablated dendritic actin arrays associated with lamellipodia and likewise impaired haptotaxis (Wu et al., 2012). These results concordantly indicate the importance of actin-based protrusion in ECM haptotaxis, with adhesion complexes serving both mechanical and signaling roles. By the same token, neither depletion of fascin-1 nor of Arp2/3 complex impaired PDGF chemotaxis, at least not significantly so, suggesting that mesenchymal cells utilize diverse, context-dependent mechanisms for achieving asymmetric force generation (Bear and Haugh, 2014). This does not discount the role of lamellipodia and lamellipodial branching in exploratory motility that allows fibroblasts to align their direction of migration with a PDGF gradient (Welf et al., 2012). Further, considering that our PDGF chemotaxis assay was conducted under near-optimal gradient conditions, our results do not exclude the possibility that coordination of bundled and dendritic F-actin plays a more important role in sensing suboptimal gradients.
Beyond this level of understanding, our results suggest a more refined hypothesis of mesenchymal taxis. A distinct aspect of haptotaxis is that the cell must actively protrude in order to encounter immobilized cues, whereas soluble chemoattractants are encountered passively, i.e., by diffusion. The search for adhesive ligands is inherently an exploration, with F-actin bundles apparently directing, and lamellipodia propagating, the process. Our observations of adhesions forming beneath the bundles, even before the emergence of lamellipodia, lead us to speculate that the two F-actin structures dynamically cooperate in the haptotactic sensing of ECM ligands, and that integrin-mediated signaling integrates those dynamics. Although this is an appealing model, this mechanism might be only one of multiple ways that fascin influences random and haptotactic cell migration. For example, fascin has been shown to regulate tension in stress fibers and thus the dynamics of mature adhesions (Elkhatib et al., 2014).

Fascin expression has been implicated in cancer progression and associated with poor prognoses in many cancers, making it a promising therapeutic target or biomarker (Arjonen et al., 2011; Machesky and Li, 2010). Fascin is associated with the epithelial-to-mesenchymal transition (Li et al., 2014) and is thus linked to acquisition of tumor cell invasiveness. Our results implicate fascin and F-actin bundles in exploratory morphodynamics that are generally important for directed cell migration, haptotaxis especially. ECM gradients are encountered during collective fibroblast invasion of wounds and likewise during tumor cell invasion of the surrounding matrix, and it will be interesting to investigate the relative roles of haptotactic and chemotactic cues in those invasion processes (Bear and Haugh, 2014; Lara Rodriguez and Schneider, 2013). Another important
aspect of haptotaxis in need of characterization is how it is affected by the structure of ECM in 3D versus 2D and contact guidance of cell migration that is a manifestation of ECM remodeling by mesenchymal cells (Even-Ram and Yamada, 2005).

3.4. Materials and Methods

3.4.1. Cell Culture, DNA Constructs, and Other Reagents

NIH 3T3 mouse fibroblasts (American Type Culture Collection), IA32 mouse embryonic fibroblasts (MEFs) (Cai et al., 2008), and Arpc2 conditional knockout fibroblasts (Rotty et al., 2014) were cultured in DMEM supplemented with 10% FBS and 1% PSG (Life Technologies). Transient transfection by lipofection was performed using either Lipofectamine Plus (Life Technologies) or Nanojuice (EMD Millipore). The EGFP-AktPH-C1 and mCherry-AktPH-C1 constructs were described previously (Weiger et al., 2009). The construct containing EGFP fused to human fascin-1 was a kind gift from J. Adams (University of Bristol, Bristol, UK) (Adams and Schwartz, 2000). To construct the mCherry-fascin-1 plasmid, the mCherry sequence was substituted for EGFP by standard methods and verified by sequencing. The tdTomato-GPI was created from the pMCP-GPI vector (NEB). The GFP-paxillin vector was obtained from Addgene (plasmid # 15233) (Laukaitis et al., 2001), as was RFP-zyxin construct (plasmid # 26720) (Bhatt et al., 2002). The EGFP-MyoX fusion construct was gift from R. Cheney (UNC-Chapel Hill) (Berg and Cheney, 2002) and the mCerulean-PA-Rac (N-term2) was a gift from the laboratory of K. Hahn (UNC-Chapel Hill) (Wu et al., 2009). The pharmacological inhibitors CK666, PP2, and FAK inhibitor II (PF-573228) were purchased from Calbiochem; PI3Kα inhibitor IV was purchase through
EMD. Antibodies for immunoblotting were obtained from Millipore (fascin mAb clone 55K2) and Cell Signaling Technologies (GAP-DH, mAb #5174).

3.4.2. RNA Interference

Two shRNAs directed against murine fascin-1, expressed in the pLKO-puro vector, were selected among several from the Lenti-shRNA Core Facility at UNC-Chapel Hill; efficacy was tested by standard immunoblotting. To co-express both of the shRNAs, puromycin resistance was swapped for hygromycin resistance in the shRNA #2 plasmid. The shRNAs were packaged in 293T cells co-transfected with pCMV-VSV-G (Addgene, #8454) and pCMV DR8.91 plasmids. The control plasmid containing a non-targeting shRNA sequence, pLKO-shNEG-puro, and pCMV DR8.91 were kind gifts from R. Everett (MCR-University of Glasgow Centre for Virus Research, Glasgow, UK). Virus was harvested, and target cells were infected by the standard method with addition of 5 μg/mL polybrene. Cells expressing shRNAs were selected using 2 μg/mL puromycin and, in the case of co-transfection, 60 μg/mL hygromycin.

3.4.3. Live-Cell TIRF Microscopy

Prism-based TIRF microscopy was performed as previously described (Johnson and Haugh, 2013; Schneider and Haugh, 2004, 2005). The microscope uses a modified Axioskop 2 FS equipped with 40X 0.8 N.A. and 60X 1.0 N.A. achroplan water dipping objectives (Carl Zeiss) and an ORCA ER CCD camera (Hamamatsu Photonics). Coverslips or glass-bottom dishes (Matek) were coated with 10 μg/mL fibronectin (BD Biosciences), except where noted
that the surface was coated with poly-D-lysine (70-150 kD, 1 mg/mL, Sigma Aldrich). Cells were briefly trypsinized and resuspended in imaging buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, and 2 mg/ml fatty acid–free BSA, pH 7.4) supplemented with 1% PSG and either 1% (NIH 3T3s) or 10% FBS (IA32s). The cells, transiently transfected one day before the experiment, were allowed to spread for 2 hours (NIH 3T3s) or overnight (IA32s). Fluorescent proteins were excited using the following lasers: 442 nm (TFP), 488 nm (EGFP), and 561 nm (mCherry, tdTomato); emission filters were 480/40, 515/30, and 630/60, respectively. Photo-activation of Rac was performed using a mercury arc lamp as previously described (Welf et al., 2012). Image acquisition was programmed using Metamorph software (Universal Imaging). The frequency of image acquisition varied from 1-12 frames per minute (most commonly, 2 frames per minute); acquisition for the protrusion dilation wave experiments (Fig. 3.12) was consistently at the upper end of the range (6 or 12 frames per minute). All experiments were performed at 37°C.

3.4.4. Morphodynamic Analysis

All image analysis was performed using MATLAB (Mathworks). Images were segmented either automatically by k-means clustering or manually via thresholding. Spatiotemporal mapping and the identification of morphological extensions were performed as described in detail previously (Johnson and Haugh, 2013; Welf et al., 2012). Briefly, protrusion/retraction for the spatiotemporal maps was calculated by the difference in overlap between consecutive frames. The angle of each protruded/retracted pixel relative to the cell
centroid is determined, and these are binned according to 1-degree increments. The sum of the pixel counts in each bin for the time interval is converted to a velocity, which is plotted as a function of angle and time and smoothed with a moving average filter. To identify morphological extensions, the distances of all pixels on the cell contour from the centroid are first measured; portions of the contour that exceeded the moving average distance taken over 100-pixel windows for more than a 13-μm contour length, and which contained at least 50 pixels in the extended region, were designated as extensions.

3.4.5. Analysis of Lamellipod/Filopod Overlap

Lamellipod/filopod overlap was analyzed by segmenting filopodia based on geometry and lamellipodia based on continuous regions of protrusion. Spatiotemporal angular maps of both these structures and the sequence of overlap was determined. For further details see sections 4.2.6 and 4.2.7

3.4.6. Ratiometric Analysis of PI3K Signaling in Filopodia

The volume marker (TFP) channel was used to make masks of the putative filopodia as described in the previous section. For each filopod, the base was identified as the end connected or closest to the cell body. Tracking of filopodia across frames of a movie was carried out as follows. If the base of a filopod was within a few pixels of one found in the previous two frames, it was counted as the same; otherwise, it was counted as a new filopod. Filopodia that appeared for fewer than 3 frames were considered non-adherent and removed. The aforementioned mask of the cell excluding filopodia was eroded by 5 μm to identify a
central region of the cell. The mean, background-subtracted TIRF intensities, F, from the TFP and AktPH channels were calculated for both the filopodia and the central region. For each filopod, the enrichment ratio, E, was calculated and averaged over its lifetime.

\[ E = \frac{F_{\text{AktPH,filopod}}}{F_{\text{TFP,filopod}}} / \frac{F_{\text{AktPH,central}}}{F_{\text{TFP,central}}} \]

A value of \( E > 1 \) is interpreted to mean that AktPH is enriched in the filopod relative to a cytosolic volume marker. See also section 4.2.8

3.4.7. Quantification of Change in PI3K Signaling Pattern

To quantify the change in PI3K signaling in response to FAK inhibition, we first identified morphological extensions as described under Morphodynamic analysis above. Subtracting these regions from the whole-cell mask yields a mask of the ‘cell body’. The mean AktPH intensity in the cell body region was used as the background level, which was subtracted from the highest AktPH mean intensity among the morphological extensions. The net intensity thusly calculated, averaged over 5 frames beginning 5 minutes after adding FAK inhibitor was compared as a ratio to the net intensity for the 5-frame average just before the inhibitor was added (after/before). Complete ablation of localization (AktPH intensity becomes spatially uniform) by the inhibitor returns a value close to zero, whereas no change in pattern of localization returns a value of 1.
3.4.8. Chemotaxis and Haptotaxis Assays

Microfluidic devices were prepared as previously described (Wu et al., 2012). Briefly, transparency masks were printed using a high-resolution printer (Fineline Imaging, Colorado Springs, CO), and the pattern for the chamber was fabricated on 4” silicon wafers by a two-step photolithography process. The silicon wafer was exposed to silane overnight after developing and postbaking. Polymethylsiloxane (PDMS) was then poured on the wafer and cured overnight at 70°C. Individual PDMS devices were cut out from the wafer, and ports were punched out. The devices were washed with water and then with ethanol, blow-dried, and plasma-cleaned. The PDMS device was placed into contact with a glass dish bottom immediately, ensuring that an irreversible seal was formed. To establish a PDGF gradient, 27 ½ gauge needles were attached to gas tight 100 µL Hamilton glass syringes (81020,1710TLL 100µl SYR) and connected via tubing to the entrance ports of the device. Both syringes were filled with DMEM, and the source was supplemented with 120 ng/mL PDGF and 1 - 5 μg/ml TRITC/Cy5-dextran to visualize the gradient. Using a syringe pump, the flow rate was set to 20 nL/min and a stable gradient was established within 30 minutes. Haptotactic gradients were formed by addition of 250 μg/mL Cy5-labeled fibronectin to the source channel. After washing with sterile PBS, the gradient of immobilized fibronectin was visualized by epifluorescence prior to seeding cells in the culture chamber.

Co-cultures of control and fascin shRNA-containing cells were used in each chamber. The control cells were dyed using Celltracker green (Life Technologies), with the exception of the rescue experiments, in which the rescue cells expressed EGFP-tagged human fascin-1 (isolated by fluorescence-activated cell sorting). Differential interference contrast images
were acquired every 10 minutes for 12-24 hours using a Olympus IX81, Olympus VivaView FL, or Nikon Biostation microscope, each equipped with motorized stages and a 20x SAPO 0.75 N.A. (0.5 magnification) or a 20x plan fluor DL 0.5 N.A objective. All experiments were performed at 37°C in a humidified environment.

Cells were tracked using the manual tracking plugin (Ibidi) in ImageJ. Intervals during which cells were touching or dividing were excluded. The cell centroid tracks were analyzed in MATLAB to calculate forward migration index (FMI), speed, persistence (D/T ratio), and to construct wind-rose plots. Each wind-rose plot shows a histogram of the angles between endpoints of each track, smoothed by a moving average. The FMI is defined as the total translocation in the direction of the gradient divided by the overall path length (Foxman et al., 1999). The D/T ratio is defined as the Euclidian distance between endpoints divided by the overall path length.

3.5. Conclusions & Outlook

3.5.1 Conclusions

Here we have described a mechanism through which F-actin bundles/filopodia containing fascin-1 serve as templates for formation and orientation of lamellipodia. Accordingly, modulation of fascin-1 expression tunes cell shape, quantified as the number of morphological extensions. Ratiometric imaging reveals that F-actin bundles/filopodia play both structural and signaling roles, as they prime the activation of PI3K signaling mediated by integrins and focal adhesion kinase. PI3K signaling is sustained through positive feedback through adhesions and arp2/3 mediated dilation of protrusions. Depletion of fascin-
ablated fibroblast haptotaxis on fibronectin but not PDGF chemotaxis, consistent with the previously reported effects of depleting Arp2/3 complex. We conceptualize haptotactic sensing as an exploration, with F-actin bundles directing, and lamellipodia propagating, the process, and with signaling mediated by adhesions playing the role of integrator.

3.5.2 Outlook

While much has been done to improve our understanding of how mesenchymal cells reorient in the presence and absence of directional cues, additional studies are required to fully understand the responses to directional cues and dynamics of positive feedback. Advancements in tools to locally perturb adhesion formation will be necessary to more firmly establish the feedback dynamics on PI3K through adhesions. Knockdown and mutant studies will be needed to parse the contributions and contexts which FAK and Src individually contribute to PI3K signaling. Additional work that includes high resolution imaging of actin bundle dynamics during haptotaxis is needed to determine if haptotactic sensing is dictated by probabilistic adherence of actin bundles. While fascin was not required to chemotax in an ideal setting, addition experiments would be needed to determine if actin bundles affect the ability to chemotaxis in shallow and non-ideal gradients. Although we characterized the effect of fascin depletion in the context of directional cues, the effect of fascin overexpression on tactic fidelity also remains unknown. Studies addressing the role of actin bundles or fascin in the context of durotaxis or chemotactic ligands affixed in a gradient to the surface would provide crucial data to understanding which sensing roles actin bundles can play. The later would be of particular interest as it bridges the gap between haptotaxis
and chemotaxis. To delineate the effect of fascin outside of its role of actin bundling in directed cell migration studies using actin-binding deficient mutants would be required. The role of other bundling proteins in actin bundle guided lamellipodia formation and directed migration still needs to be investigated. Finally, it would be of interest to know if these mechanisms persist in amoeboid cells or 3D/in vivo environments, and if not, what parallels can be drawn between the mechanisms described in mesenchymal cells.

3.6. Acknowledgements

We are grateful to colleagues who provided plasmids as indicated under Materials and Methods. We also wish to thank Erik Welf and Keefe Chan for helpful discussions and Elizabeth Haynes for providing Arp2/3 microinjection movies. This work was supported by National Science Foundation grant #1133476 (J.M.H.) and National Institutes of Health grant GM083035 (J.E.B.).

3.7. References


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CHAPTER 4

Quantitative Image Analysis of Cellular Morphodynamics and Signaling

4.1. Introduction

Automated image analysis has been a goal for many applications since the advent of digital images. It is used in many applications within computer vision from robotics to facial recognition (Desouza and Kak, 2002; Yang et al., 2002). More recently, it has used in an attempt to develop high-throughput diagnostic assays (Brennan et al., 2010; Cheng et al., 2010). Another application for image analysis is in obtaining quantitative data from images, often of biological specimens, for the purposes of research. For many years imaging data of cellular phenomena was largely qualitative. In the last 10 years the advent of genetically encoded fluorescent proteins and demand for more quantitative data in cell biology has made the use of automated image analysis for cell biology applications increasingly attractive. Image analysis is more efficient, consistent, and complete than manual analysis of image data (Dorn et al., 2008). Simple quantification by counting based on a defined criteria can be sufficient for more obvious phenomena, but subtle and emergent features require a much more robust and unbiased approach. This factor combined with the falling cost of computational power has made the use of computer vision an ideal tool for extraction of quantitative results from micrographs.

In this chapter we develop several techniques for obtaining quantitative data from live cell images collected using several different contrast methods included both reflected and transmitted light images. These analyses include both cytoskeletal and signaling structures, sometimes in multiple channels. The output from these analysis techniques have proved invaluable in obtaining the results shown in the previous two chapters as well as other various published studies (Asokan et al., 2014; Rotty et al., 2014).
4.2. Analysis Techniques

4.2.1 Basic Image Processing and Segmentation of Cells

All of these techniques have been implemented in MATLAB but could be extended to other platforms. In order to perform subsequent analyses typing the cell must be segmented from the background first, for fluorescence images this is usually relatively simple and can be done by a variety of methods. Segmentation of other contrast methods is more challenging. For segmentation of phase or DIC images refer to section 4.2.15. We most commonly use k-means clustering or simple thresholding to achieve this. K-means clustering is a useful tool which bins the image in a way which minimizes the variance of each bin. The number of bins is adjustable and it can be used to pick out various regions of similar intensity. The one drawback is that it is a somewhat slow calculation, particularly with a large number of bins. An alternative faster method is simply to threshold the image based on the intensity at the inflection point when performing a linescan from the background to cell. The drawback to this is it must be set manually each time there is a change in the background levels. Figure 4.1 shows the example of preparing and segmenting a cell for further analyses. A more detailed protocol for basic segmentation of fluorescent cells can be found in appendix B1.
Figure 4.1. Basic image processing and segmentation.  a) Unprocessed TIRF image of randomly migrating NIH 3T3 fibroblasts expressing GFP-AktPH. Scale bar = 20 μm.  b) Cropped, rescaled, and pseudocolored image from a. The image has been cropped to contain the migration path of a single cell throughout the time course. The dark square in the top left corner of the field shows where another cell entering the region at a later time has been removed. Scale bar = 20 μm.  c) Above: mask of the cell in b obtained by identifying the first bin of the k-means segmentation as the background. Below: mask of the cell (blue) with hot spot regions overlayed (red).
4.2.2 Segmentation of Signaling Hot Spots

In order to perform spatial signaling analyses it is sometimes helpful to segment signaling enriched regions. This segmentation has been used previously for PI3K activity (Melvin et al., 2011; Weiger et al., 2010) (see also appendix B.2) and more recently applied DAG (diacylglycerol; Asokan et al., 2014) and Arp2/3 activity (Rotty et al., 2014). Identification of these regions by segmentation (Fig. 4.1c) enables several forms of analysis that can provide further insight into spatiotemporal dynamics of signaling. This process is relatively straightforward but can be accomplished by two different methods. One method is to use a higher bin from k-means segmentation which may have already been used to segment to the cell from background anyway. Another less computationally intensive method is to simply use contiguous regions in which the signal is elevated a set number of standard deviations above the mean cellular intensity. One potential pitfall of this method is that sometimes apparent signaling regions will become segmented when the signaling is globally low. This is due to the fact that if the signal is relatively uniform, then the standard deviation will be low. However, if one can be assured that this will not be an issue with the current images this provides a faster and more easily tunable method for signaling segmentation.

4.2.3 Calculating a Macroscopic Signaling Vector

With signaling hot spots segmented one can calculate the macroscopic signaling vector. The macroscopic signaling vector is a weighted sum of the vectors drawn from the centroid of the cell contact area to those of the aforementioned signaling hot spot regions.
identified by segmentation in 4.2.2 (Weiger et al., 2009, 2010) (Appendix B.3). Having both a direction and a magnitude, the vector is an appropriate metric representing the overall asymmetry/anisotropy of the biosensor translocation pattern. We have demonstrated how dynamics of the signaling vector may be analyzed, manifest as a wind rose plot (Weiger et al., 2009), as a correlation with the cell centroid displacement vector (Weiger et al., 2010) (Fig. 4.2a), and as a path with successive signaling vectors joined head to tail (Melvin et al., 2011).

4.2.4 Hot Spot Tracking

Although calculation of the macroscopic signaling vector is relatively simple to implement, one can only infer dynamical information from it by assessing changes in the signaling vector as a function of time. This led to the development of a method for tracking individual hot spots and determining their fates (Weiger et al., 2010) (Fig. 4.2b; Appendix B.4). It uses the same data already curated 4.2.3. The algorithm starts with a hot spot region in the previous image and checks for spatial overlap with a hot spot, or multiple hot spots, in the next frame(s). If only one such hot spot is found, it is identified as the same hot spot as in the previous image. If none is found, then the hot spot is declared dead. If instead two (or more) hot spots are found, the “new” hot spots are said to have been born by branching and are linked historically to their progenitor. Carrying out this operation in reverse identifies instances when two (or more) hot spots merge into one. Finally, any hot spot not yet accounted for is considered to have been born spontaneously.
Figure 4.2. Advanced image analysis methods.  

a) Construction of the macroscopic signaling vector ($S_R$) as outlined 4.2.3. If the goal is to correlate $S_R$ with the centroid movement vector, $C$, averaging over larger time intervals may be warranted, depending on the temporal resolution of the movie ($\Delta t$) in relation to the cell movement speed. 

b) Hot spots were tracked from birth to death as outlined in 4.2.4. Their centroid paths are plotted as connected lines, with elapsed time color-coded from cool to warm.

c) Montage of the cell depicted in Fig. 1 (left) and the accompanying spatiotemporal signaling map (right), created as in 4.2.5. Times shown on the montage correspond with those on the map. Scale bar = 20 μm.
4.2.5 Constructing a Spatiotemporal map of Signaling and Morphodynamics

A method was developed to visualize signaling, protrusion, and retraction with respect to location and time in a single cell (Welf et al., 2012) (Fig. 4.2c; Appendix B.5). Using either protrusion data from cell mask overlap or signaling data which may or not be pre-segmented these values can be mapped radially about the cell. This approach is similar to kymograph-like methods used to visualize cell motility dynamics (Machacek and Danuser, 2006). Originally used on PI3K signaling it can be adapted for various types of signaling or any segmented structure (Asokan et al., 2014). The cells center must be excluded to prevent artifacts caused by pixels being too close to the centroid. If not segmented, signaling is normalized by the number of pixels in each angular bin to prevent artifacts caused by elongated cell morphologies.

4.2.6 Segmentation of Filopodia

For analysis used in chapter three it was necessary to develop an algorithm to segment and dynamically track filopodia throughout their lifetimes. To identify putative filopodia in each cell, the area containing the cell was cropped and binarized by a manually set threshold. This threshold was determined by starting from a low value and iteratively increasing it until either the cell outline becomes sharp or filopodia begin to be lost; at that point, the threshold is returned to the lowest value that gives a reasonably crisp cell outline. A seven-pixel wide (1.75 μm at 40X), square top-hat filter was applied to the binary cell mask to segment filopodia. Pixels were filled in between segmented structures within a few pixels of each other to ‘heal’ discontinuities. Then, regions below a certain area cut-off (less
than 15 pixels at 40X) were removed. By subtracting the resulting mask of segmented filopodia from the whole-cell mask, a mask of the rest of the cell was also generated. The first several frames of each cell were checked to ensure reasonably accurate segmentation. A sensitivity analysis was performed on a few cells, varying the chosen threshold and the constant top-hat filter size. Increasing or decreasing the threshold by 20% of the chosen value or varying the filter size by ± 1 pixel altered the mean number of filopodia identified by no more than 15% across all frames for the cells tested.
Figure 4.3. Automated identification of filopodia. (a) Putative filopodia are segmented (red; cell mask shown in yellow) by top-hat filtering of TIRF images. These structures may be tracked based on frame-to-frame overlap/proximity. (b) The structures thusly identified tend to be enriched in FP-tagged fascin-1. (c) The putative filopodia are labeled by FP-tagged Myosin X at their tips. Scale bars = 10 μm.
4.2.7 Analysis of Lamellipod/Filopod Overlap

Based on the morphodynamic characteristics of lamellipodia as seen in protrusion maps (Machacek and Danuser, 2006), we defined these structures as continuous regions showing estimated protrusion velocity > 1.25 μm/min and spanning at least 8 degrees for at least 1 minute. This is done using the mask excluding filopodia; therefore, the pixels associated the identified lamellipodia and filopodia cannot overlap, rather, we define the regions as ‘overlapping’ if they contain pixels in the same angular bin or in adjacent bins (for the same frame of the movie or in adjacent frames). For the FP-fascin-expressing cells, submembranous bundles were included in addition to the putative filopodia. Those bundles were segmented from the FP-fascin channel by applying a 1 μm top-hat filter and taking the highest k-means bin of that image. Bundles not within 5 μm of the cell edge were excluded.

4.2.8 Ratiometric Analysis of Sub-diffraction Structures

To investigate signaling within filopodia, it was necessary to combine segmentation, tracking, and ratiometric imaging. Although the signal cannot be clearly resolved due to diffraction of light and a pixel size larger than the structure itself, the light from the structure can still be collected quantitatively even though it may be diluted across several pixels. Varying distance from the coverslip poses an additional issue, as the power of TIRF illumination varies as a function of distance.

To subvert these issues, we segmented and track filopodia and ratioed the intensities of the membrane localized signaling marker with a soluble fluorescent protein. We used the filopodia segmentation method described above in section 4.2.6 and tracked filopodia as a
function of time using their overlap and proximity between frames. It was necessary to track them to avoid multiple counts of the same filopodia. Due to the fact that the focal distance varies slightly between different wavelengths of light, we dilated the filopodia masks in order to capture any fluorescence which had spilled over into neighboring pixels due to the small focal difference. Using a fluorescent labeled membrane marker, we can see that the amount of membrane within filopodia in the illumination area is also increased making it difficult to delineate the contributions of membrane and activity enrichment. (Fig. 4.4)
Figure 4.4. (a) Putative filopodia were identified from volume marker (TFP) images as described under section 4.2.6. The mask associated with filopodia is dilated, and the mean intensity of each filopodial region is computed along with that of the cell interior. The ratio of those intensities (filopod/interior) is recorded. This procedure is then repeated for each region of pixels in the other channel, the mCherry-AktPH biosensor, for example. In this case, the enrichment ratio, E (the filopod/interior ratio of AktPH normalized by that of the volume marker) is used to assess the presence of PI3K signaling in the filopodia. (b) The value of E reflects both the extent of biosensor translocation (from the cytosol to the membrane) and the filopod geometry, effects that are not readily decoupled. Using a glycophosphatidylinositol (GPI)-anchored FP to uniformly label the outer leaflet of the plasma membrane, we estimated the relative enrichment of plasma membrane area relative to the volume of a filopodium. With FP-GPI in place of FP-AktPH, the mean value of E was 4.7 (n = 798 filopodia from 9 cells; error bars indicate ± 95% confidence interval), indicating a significant effect of geometry. Put another way, the ratio of AktPH/volume marker TIRF should not be used to compare relative 3’ phosphoinositide densities in filopodia versus elsewhere in the contact area.
Using the masks created with the soluble marker an enrichment ratio, $E$ was calculated for each structure. This ratio would represent the enrichment of the signaling probe in that structure relative to in the cell body and was defined as the ratio of the signaling in the structure with the cell body compared to the same ratio calculated with the cytosolic marker.

$$E = \frac{F_{AkPH, filopod}}{F_{AkPH, central}} \div \frac{F_{TFP, filopod}}{F_{TFP, central}}$$

An $E$ equal to one would indicate that the enrichment of the probe in the structure is the same as the enrichment of the cytosolic marker, hence that it is not enriched. Values greater than one indicate an enrichment of the signaling probe within the segmented structure.

### 4.2.9 Cellular Extension Segmentation and Counting

As cell migration is frequently dependent on activity which is occurring within the lamellipodium, it is often desirable to restrict analysis to this region. While the “lamellipodia” identified in the aforementioned overlap analysis require active protrusion to be counted we desired to count regions which did not necessarily have continuous protrusion or from static images from which protrusion cannot be identified. This led us to develop a way to segment the morphologically extended parts of the cell which often include PI3K signaling. However, this method does not distinguish between actively protruding regions and therefore often includes the “tail” of a migrating cell as well.

To accomplish this, the cell periphery is established from the cell mask and the local mean is calculated for each point along it. Contiguous areas of perimeter of sufficient size,
and length, and breadth can be separated out and counted as extended areas. The precise criteria for each of these requirements will depend on the cells, purpose of analysis, and magnification/resolution of the images. Analysis can be restricted to these segmented areas or the number of regions can simply be counted as in **Fig 3.5.** One can also calculate the distribution of sizes and shapes of these extensions and compare groups of cells under different criteria. This type of analysis was performed for a LKB-1 deficient melanoma cell line and its rescued counterpart as well as a microtubule affinity-regulating kinase (MARK) dominate negative expressing version to search for subtle differences in morphology (**Fig 4.5**) (Chan et al., 2014).

**Figure 4.5.** Examples of extension analysis to profile changes in cell morphology across subgroups. Analysis of extension breadth and total extended area was quantified for many cells for 300 timepoints over 2.5 hours. The analysis was performed for a LKB-1 null melanoma cell line as well as LKB-1 addback and MARK dominate negative version. Grey dots represent the mean values for individual cells over the timecourse. Blue lines ± standard deviation. Red boxes indicate 95% confidence intervals and the red lines indicate the mean.
4.2.10 Analysis of DAG Signaling

In a study of myosin signaling pathways relation to chemotaxis it was necessary to analyze angular bias of diacylglycerol (DAG) in response to a PDGF gradient in migrating fibroblasts (Asokan et al., 2014). A fluorescently-tagged tandem C1 domain probe which binds to DAG was imaged using TIRFM to track DAG localization. Several cells were imaged and agglomerate plots of signaling bias were created, for both PDGF chemotaxis (Fig 4.6a) and the random migration control (Fig 4.6b).
Figure 4.6. (a) Enriched pixels in a chemotaxing cell are shown outlined in magenta. The intensity values of the segmented pixels are summed within angular bins at each time point to create a signaling “map” with the angle plotted on the horizontal axis and time on the vertical axis. Histogram showing the cumulative intracellular DAG distribution in chemotaxing cells expressing (C1)2-GFP (n = 12 cells). (b) Enriched pixels in a randomly migrating cell are shown outlined in magenta. The intensity values of the segmented pixels are summed within angular bins at each time point to create a signaling “map” with the angle plotted on the horizontal axis and time on the vertical axis. Histogram showing the cumulative intracellular DAG distribution in randomly migrating cells expressing (C1)2-GFP (n = 13 cells).

To accomplish this, spatiotemporal maps of signaling were created somewhat similar to as in section 4.2.5. A mask of the cell is created via thresholding and removing small isolated groups of pixels (Fig 4.7 (1)). A mask of the cell periphery is created from the whole
cell mask by erosion of edges and subtracting the resulting image from the cell mask (Fig 4.7 (2)). Locally enriched signaling regions are determined in the periphery mask by segmenting contiguous regions (at least 15 pixels) with intensity values that exceed a set number (1.2) of standard deviations above the mean intensity of the periphery mask (Fig 4.7 (3)). Any pixels completely enclosed by enriched pixels are also included (outlined in magenta). As in section 4.2.5, the intensity values of the segmented pixels are summed within angular bins relative to the cell centroid in each image. This is done for each time point to create a signaling “map” (Fig. S5E(4)) with the angle plotted on the horizontal axis and time on the vertical axis. To quantify the overall signaling pattern for each cell, the values in each angular bin is averaged over all time points and then normalized such that the mean across all bins is equal to 1. These normalized patterns are then averaged over all the cells for each condition to produce an aggregate plot. The linear (unbinned) plots are smoothed by local regression using weighted linear least squares and a first-degree polynomial mode. The cumulative DAG signal is then presented as a histogram with a bin size of 10 degrees for chemotaxing cells ($n = 12$ cells) and randomly migrating ($n = 13$ cells) (Fig. 5E, 5F). A similar approach was used to analyze the distribution of pSer19 of RLC in chemotaxing cells.
Figure 4.5. Method of analysis of the localization of DAG intensity in cells transfected with (C1)2-GFP. (Top) Original pseudocolored image is shown in the first panel followed by the cell mask, and peripheral region. (Bottom) Segmented enriched peripheral signaling, (bottom right) Signaling map from the cell shown.

4.2.11 Analysis of Periphery Arp2/3 Signaling

Often, it is desirable to look at signaling that occurs at the very edge of the lamellipodium, or to exclude perinuclear localization of probe. Since most microscopes restrict the plane of light collected rather than what is illuminated the Z distance collected is typically much larger than with TIRF this can increase the amount of perinuclear signal
observed. In this case cells were fixed and stained for Arp2/3 complex and which is known to localize to the edge of the lamellipodium (Fig 4.8) (Rotty et al., 2014).

After segmenting the cell from background as described in 4.2.1, the elevated signaling regions can be segmented. Similar to the analysis of DAG signaling, these regions can be segmented based on standard deviations from the mean or k-means. The majority of perinuclear and background localization can be removed by simply restricting the signal to a band around the edge of the cell of similar width as the observed localization. This operation can be performed by dilating the perimeter of the cell by the desired amount and restricting signaling to this peripheral mask. The remaining segmented signal can then be binned angularly if desired and combined across multiple cells if analysis of angular bias is desired such as in section 4.2.11 (Fig 4.8). Alternatively, the fraction of the cell edge that is enriched with signaling can be calculated based on the total number of pixels within the edge region (Rotty et al., 2014).
**Figure 4.8.** Arp2/3 complex edge ratio: (Top left) Cell stained for arp2/3 complex. (Top Right) High Arp2/3 complex signal designated as intensity greater than 1.2 standard deviations above the mean signal for the whole cell. (Bottom Left) High signal restricted to a 5 pixel band along the cell perimeter. (Bottom Right) Angular distribution of Arp2/3 enrichment along the perimeter of the cell.

### 4.2.12 Segmentation and Quantification of MLC Puncta

As with the distribution of DAG signaling in section 4.2.11 it was necessary to analyze the distribution of myosin II regulatory light chain (RLC) in response to PDGF gradients with both a WT RLC and a dominate negative mutant (S1AS2A) (Asokan et al., 2014). However, unlike the previous analysis it could not be based around the simply intensity of myosin regulatory light chain (RLC) due to the fact that it localizes strongly to stress fibers and only punctate localization was of interest. To remedy this it was necessary
to first differentiate myosin “puncta” from stress fibers. The WT RLC formed puncta in the direction of the gradient with stress fibers primarily forming primarily perpendicular to the gradient (Fig. 4.9a). Conversely the mutant did not appear to organize in a particular fashion and often had more stress fibers (Fig. 4.9b). RLC localization in response to a gradient of the myosin inhibitor blebbistatin (BLB) was also analyzed; however, due to the myosin inhibition, stress fibers were mostly absent, and the analysis described under section 4.2.11 could be used (Fig. 4.9c).
Figure 4.9. (a) TIRF microscopy movies of chemotaxing MyoRLC-GFP cells are analyzed to identify puncta (blue) and stress fiber regions (red). The localization of puncta and stress fibers relative to the cell centroid were averaged across multiple cells (n = 11 cells) and presented as histograms. (b) Analysis of puncta and stress fiber distributions, quantified as in (A), in MyoRLC(S1AS2A)-GFP cells (n = 36 cells). (c) Circular histograms of MyoRLC-GFP (n = 162) and MyoRLC(S1AS2A) (n = 67) cells chemotaxing in a gradient of BLB. (d) Intensity of MyoRLC-GFP and MyoRLC(S1AS2A)-GFP during chemotaxis in a BLB gradient, presented as a histogram.
Accordingly, masks of the cell and cell periphery are created. Due to the obscured visibility and discontinuity of the cell outline in cells expressing RLC-GFP constructs, a convex hull around the original cell mask is used to calculate the cell centroid as well as to exploit the fact that stress fibers reside almost exclusively in concave regions. An example of stress fibers and puncta are shown in Fig. 4.10a. Using a two-dimensional median filter (Fig. 4.10b (1)), puncta and stress fibers in the outer ring of the convex hull mask are segmented. A larger geometry and lower threshold is used for segmenting stress fibers due to their larger size. Median-filtered objects are organized by size, objects of only a few pixels are removed, and large objects with high aspect ratios are identified as stress fibers (any other large objects are removed). If a stress fiber is identified in one frame, median segmented regions that overlap with it in the next frame are also considered stress fibers if they have a high aspect ratio. This aids in the detection of stress fibers that might not be fully segmented by the median filter in every frame. Each of the pixels associated with puncta is assigned to an angular bin with respect to the cell centroid (1 count per pixel); any bin containing both puncta and stress fibers are counted as stress fibers. To correct for discontinuous stress fibers missed between frames, any angular bin marked as containing stress fibers more than half the time during a 15-frame interval is marked as a stress fiber for the entire interval. After this procedure, each angular bin is classified as puncta, stress fiber, or neither. To plot the distribution of puncta, stress fiber bins are set to zero, and vice-versa for the stress fiber distribution. The plots are generated in the same manner as described above for the DAG pattern. The data for the wildtype RLC-GFP-expressing cells in the PDGF gradient are shifted by the median angle of migration (not including angles more than 90° from the
direction of the PDGF gradient), resulting in a 25-degree shift to the right. During migration in response to a BLB gradient, very few stress fibers were visible under TIRF imaging. Analysis was therefore performed in the same manner as for the DAG pattern to quantify RLC-GFP regions at the cell periphery. Examples of signaling maps generated in the BLB gradients are shown in Figure 4.10c.
Figure 4.10. (a) Image of a chemotaxing MLC-GFP cell. Magnified sections show myosin organization with puncta in the blue box and stress fibers in the red box. (b) A two-dimensional median filtering (1) is used to segment, puncta (2) and stress fibers (3) in the outer ring of the convex hull mask. Puncta are marked as blue and Stress fibers as red in the final segmentation (4). (c) The intensity values of the enriched segmented pixels in MyoRLC-GFP and MyoRLC(S1AS2A)-GFP cells chemotaxing to BLB gradient are summed within angular bins at each time point to create a signaling “map” with the angle plotted on the horizontal axis and time on the vertical axis.
4.2.13 Accurate Cell Segmentation in Phase and Differential Interface Contrast Images

While segmentation of cell outlines from fluorescent image data is typically straightforward, segmentation of cell outlines from phase contrast or differential interface contrast (DIC) images is much more difficult. These contrast methods have the advantage that no marker is required in the cell and are useful for observing cell morphology and migration. For cell types which are difficult to transfect or become sick from dyes these are often the imaging methods of choice.

The primary challenge in segmentation of these images is the cell and background are often both of varying intensities and contrast levels. Cells cannot be segmented by thresholding as the cell has both higher and lower intensity regions than the background. Therefore, the gradient of intensity values are typically used to distinguish background from cell. Alternatively active contours have been used for phase segmentation (Ambühl et al., 2012; Nejati Javaremi et al., 2013). The rear of the cell often provides good contrast, however lamellipodia are thin, and often have very little contrast from the background. Debris is also much more visible in phase and DIC images and cause issues in the segmentation.

To attempt to circumvent these issues, we employed an iterative temporal correction to prevent errors from low contrast and moving debris. First an accurate outline of the first frame is obtained by manually thresholding the gradient of the image and removing the highest k-means bin. It was found that generally the highest intensity pixels occur around the edge of the cell and in the nucleus of the cell. Most thin peripheral structures such as retraction fibers can often be removed by morphological opening. In future frames if the
protrusion and retraction areas can be calculated by overlap between frames. These protrusions and retractions are checked based on size and geometry. If a protrusion or retraction appears to be an artifact caused by improper segmentation the gradient threshold is varied until the change is within the parameters. If a solution cannot be found, the boundary from the previous frame is used in the region in question. This segmentation of single cells can produce outlines accurate enough for additional analysis (Fig 4.11a) such as measurements of extensions (section 4.2.9) or protrusion/retraction maps (section 4.2.5).

In some cases, images are not high enough resolution or contrast to accurately segment cells. While they are not suitable for morphological profiling these cases, often centroid migration data can still be obtained (Fig 4.11b). Since iterative correction is computationally intensive and setting manual thresholds for each cell is not feasible, accuracy must be sacrificed to simultaneously segment many cells. In this case the gradient image is smoothed and healed and structures subcellular in size are removed. A rougher temporal correction is still employed to make sure cells are not lost between frames. Cells are tracked in the same manner as hotspots (section 4.2.4) except the merging event is no longer allowed. A branching event now represents cell division. Cells are retired from tracking if they touch the image boundary.
4.3. Conclusions and Outlook

Automated image analysis provides powerful tools for obtaining quantitative data from micrographs. Often image analysis seeks to pick out what we observe by eye, but in a much more defined, quantitative, and high-throughput manner. Segmentation of signaling and cytoskeletal structures allows for detailed comparison of phenomena across many cells. In addition to allowing us to quantify phenomena which we observe qualitatively, image analysis can also be used to pick out statistically significant subtle differences between cell populations which often cannot be realized by eye. Signaling structures can be segmented by both intensity and geometry. Segmentation of cytoskeletal structures can be performed based on morphology or using particular protein markers, as in the case of segmentation of actin bundles simply using geometric constrains or with FP-fascin. Morphological criteria can be applied to further restrict analysis of signaling, such as limiting signals to the cell periphery.
or to morphological extensions.

In order to continue to obtain quantitative data from increasingly complex, 3D, and *in vivo* micrographs advanced image analysis techniques will be required. 3D images add an additional dimension of complexity and the current methods would need to significantly retooled to work in a three-dimensional environment. Working with *in vivo* images imposes additional challenges due to image quality and interactions with unlabeled tissues. Ways to reduce noise and identify only the desired components of the image will be required. Additional work will be needed to improve the robustness of phase and DIC segmentation across varying resolutions, illumination, and image quality. Dense clusters of cells introduce the additional challenge of determining boundaries between cells.

### 4.4. Acknowledgements

I wish to thank current and former members of the Haugh laboratory, especially Shoeb Ahmed, Adam Melvin, Ian Schneider, Michael Weiger, and Erik Welf, for their contributions to the development of these protocols. This work was supported by a grant from the National Science Foundation (#1133476).

### 4.5. References


CHAPTER 5

Summary and Outlook
5.1. Summary

The main objectives of my doctoral research were to understand the role of PI3K signaling in directed cell migration and the mechanism by which mesenchymal cells make and orient new branches; to achieve these goals, we developed and refined image analysis techniques to aid quantification of signaling and morphological changes during cell migration. We have fully described a mechanism by which fibroblasts form branches that enable large scale turns, and we implicated specific roles for PI3K signaling in that mechanism (Johnson et al., 2015; Welf et al., 2012). Filopodia and fascin-containing, submembranous F-actin bundles both guide the formation of lamellipodia and prime PI3K signaling. PI3K signaling mediated by adhesions is FAK-dependent, as revealed through pharmacological inhibition studies and experiments on poly-D-lysine versus fibronectin. Adhesions form at the base and eventually along the length of filopodia to prime PI3K signaling. PI3K signaling is subsequently amplified through positive feedback from newly formed adhesions as the newly formed lamellipodium spreads/dilates. Actin bundles are often sustained throughout periodic protrusion-retraction cycles, thus maintaining the branched architectures. The formation of multiple branches allows the cell to pivot between the branches and make large-scale turns of up to 90 degrees. During PDGF chemotaxis, the branch that experiences the highest chemoattractant concentration is favored; yet, disruption of actin bundles by fascin depletion does not prevent chemotaxis. Conversely, knockdown of fascin ablates directed migration up a gradient of immobilized fibronectin (haptotaxis), suggesting that actin bundles are required for exploratory haptotatic sensing.
We have developed several image analysis methods for quantification of cell morphology, intracellular signaling, and cytoskeletal dynamics (Asokan et al., 2014; Johnson and Haugh, 2013; Johnson et al., 2015; Rotty et al., 2014). Central to these methods is the proper segmentation of images to identify discrete regions that may be analyzed, compared, and tracked. Localized regions of fluorescent biosensor enrichment are segmented using $k$-means clustering, whereas various cell structures can be segmented based on morphology. Exploiting this, we have developed protocols to segment filopodia and lamellipodia. Segmentation of morphological structures can be used for direct analysis or to restrict analysis of signaling to particular areas of a cell (e.g., its periphery). Angular mapping provides a simple and powerful way to compare signal or protrusion distributions around the periphery of the cell, and it is particularly useful in analyzing migration biased towards a particular direction, as in chemo- and haptotaxis. Probes that localize to particular cytoskeletal structures can also be used to segment and distinguish these structures. Thus, we were able to segment myosin puncta and stress fibers. Finally, we have developed an accurate time-corrective method for segmentation of phase-contrast and DIC images, which allows for additional analyses of these images.

5.2. Impact

5.2.1. The Role of PI3K Signaling in Mesenchymal Cell Migration

This work is the first to pinpoint the precise requirement of PI3K signaling in mesenchymal cell migration and turning behavior. This work contrasts with those that have implicated PI3K signaling in directed migration of amoeboid cell types, primary
*Dictyostelium discoideum*. Here, we showed that mesenchymal migration is distinct from amoeboid migration in that branching of protrusions is a stochastic process. The role of PI3K here also contrasts with amoeboid cells; we showed that surprisingly, PI3K does not dictate the number of new branches but rather their stabilization. Contrary to conventional wisdom, PI3K was not necessary for maintaining cell migration speed. We report that PI3K dynamics are strongly linked to protrusion rather than direct activation by other signals such as Rac.

5.2.2. Templating of Lamellipodia by Actin Bundles

We are the first to report that filopodia act as templates for lamellipodia in the context of migration. We speculate that this is a general mechanism, conserved across most mesenchymal cell types. This is also the first indication that PI3K signaling is enriched within filopodia in cells other than neurons. The functional requirement of fascin in fibroblast haptotaxis was also previously unknown. The finding that fascin ablation did not disrupt chemotaxis supports the idea that haptotaxis requires coordination of certain cytoskeleton structures, while chemotaxis occurs at a more basic level through myosin organization (Asokan et al., 2014). This work questions previous assumptions about the role of filopodia in migration as sensors of chemoattractants, instead suggesting a role as signaling and mechanical templates for lamellipodia and an exploratory role in haptotactic sensing.
5.2.3. Quantitative Analysis of Cell Signaling and Morphology

Image analysis methods have been central in supporting the conclusions presented in the previous two sections and have seen utility in other migration studies (Asokan et al., 2014; Chan et al., 2014; Rotty et al., 2014). Spatiotemporal mapping enabled characterization of the branching and pivoting phenomenon, and correlation of signaling with protrusion and cytoskeletal structures. Segmentation of filopodia allowed for the automatic filopodia/lamellipodia overlap analysis and ratiometric analyses. Extension analysis allowed for comparison of PI3K localization and subtle changes in morphological traits across various groups of cells. Segmentation of puncta and stress fibers from myosin-RLC data allowed quantification and distinction between distinct forms of localization of the same signal.

5.3. Outlook

While much has been done in to aid our understanding of the mechanisms that dictate cell reorientation there are still remaining questions that need to be answered. From our knowledge that fascin is required for haptotaxis we then must ask if the disruption is at the level or sensing or reorientation. Given that neither fascin nor Arp2/3 is required for chemotaxis seems to imply that the disruption is at the level of sensing which must involve both lamellipodia and filopodia. This begs the question: how do lamellipodia and filopodia work in concert to sense haptotactic gradients? The understanding gained in our study of filopodia driven lamellipodia protrusions, yields one possible hypothesis. If filopodia are produced randomly around the cell, the higher concentration of ECM ligand up-gradient
would increase the probability of a filopod coming into contact with a ligand and adhering to the substrate. This in turn, would increase the probability of producing a lamellipodia in the up-gradient. Actively protruding lamellipodia produce more filopodia, potentially further amplifying the up-gradient bias. This is a plausible mechanism as it would require both intact lamellipodia and filopodia, consistent with the previous results. To attempt to test this hypothesis one would need high resolution imaging of filopodia and lamellipodial dynamics during haptotaxis. Additionally, it would be of interest as to whether inhibition of FAK or PI3K blocks haptotaxis. The ability to locally and dynamically perturb focal adhesion formation would prove invaluable in furthering this study; unfortunately there is not currently a known means of accomplishing this. However, with recent advancements in the development of light activated kinases it might soon be possible to locally activate FAK (Gautier et al., 2011; Riggsbee and Deiters, 2010). Alternatively, methods could be developed to locally unblock ECM ligands using light (Shimizu et al., 2014). Finally, it might be possible to use optogenetics to locally cluster integrins to induce adhesion using photosensitive proteins such as cryptochrome 2 (Bugaj et al., 2013).

Recent studies show that fascin has additional roles in cytoskeletal organization apart from actin bundling (Elkhatib et al., 2014; Zanet et al., 2012). Separating the effect of fascin on focal adhesion disassembly and filopodia formation independent of actin bundling would require addition studies with mutant forms incapable of bundling. Similarly the effect of PKC phosphorylation of fascin on its role templating lamellipodia would be of interest. It has yet to be confirmed if filopodia formation is biased by internal or external cues in particular contexts, or is simply stochastic process. The precise criteria that allows some
adhesions to signaling periodically and others seeming not producing signaling needs to be better defined. Perhaps newly available superresolution techniques such as PALM, STORM, or STED could reveal the molecular differences between them. The requirement and timing of upstream Rho GTPases such as Rac during protrusion over actin bundles is similarly unclear and may tie into the differences in these adhesions.

While fascin was the focus of this study when it comes to filopodia, there are several other important components of filopodia that are necessary or enhance their formation. When we overexpressed myosin X in fibroblasts we did not observe significantly change in morphology or filopodia. Myosin X localized not only to the tips of filopodia but also to the tips of retraction fibers. However, when we co-overexpressed fascin in conjunction with myosin X the cell morphology was drastically altered, resulting in cells which contained little to no lamellipodia but numerous long filopodia (Fig 5.1). Surprisingly these cells exhibited significant motility, using clusters of extremely dynamic filopodia. The cause of this synergy is currently unknown and would be interesting to investigate in addition to the fidelity of these cells in directed migration assays. As myosin X is thought to play a role in the convergence of actin filaments in the nucleation of filopodia (Tokuo et al., 2007), perhaps fascin is a limiting component in this process, with sufficient fascin nearly all branched filaments are converged, producing this odd phenotype.
Figure 5.1. (a) An NIH 3T3 cells co-expressing: mCherry-AktPH (grayscale) and EGFP-MyoX (green) (left, representative of 15 cells in 4 experiments) exhibits normal morphology in the absence of fascin overexpression; when mCherry-fascin (grayscale) is expressed in conjunction with EGFP-MyoX (green) (right, representative of 16 cells in 6 experiments) a morphology with exaggerated filopodia and almost no lamellipodia is produced. Scale bar = 20 μm.
In addition to myosin X, formins, ENA/VASP, I-BAR domain proteins play an important role in filopodia formation. The precise requirements of these different proteins in filopodia formation and stabilization would be interesting to investigate. Some of these interact directly with downstream targets of PI3K such as myosin X which contains a PH domain and can bind to PIP₃ (Plantard et al., 2010). Recent evidence has also shown that WAVE can interact directly with ENA/VASP, giving an additional possible route of filopodia enhancement through PI3K (Havrylenko et al., 2015). The small Rho family GTPase RIF is thought to promote filopodia formation as well (Pellegrin and Mellor, 2005). However in our system constitutively active RIF failed to produce a clear effect on filopodia. This is perhaps due to a lack of significant mDia2 expression in the NIH 3T3 cell line, I would be interesting to see if RIF plays a larger role in other cell lines and if that role is critical what takes its play in the NIH 3T3 cells.

Finally, confirmation that these phenomena exist in three dimensions as well as in vivo would be of great interest. At present, most in vivo imaging techniques lack the resolution to clearly resolve individual filopodia, and pose additional challenges in that many non-adherent and dorsal filopodia would also be observed. Since in three dimensions the cell can come into contact with ECM on all sides it would be interesting to see if these dorsal filopodia serve the same functions as those on the ventral side. In vivo fascin is known to play roles in invasiveness (Darnel et al., 2009; Machesky and Li, 2010). It is possible that overexpression of fascin leads to an abundance of filopodia that disrupts direction sensing by simply overwhelming any bias that would occur do to ECM ligand concentration. While
testing this in *in vivo* would be most interesting, it would currently be possible to test this in 2D haptotactic assays.

Among the greatest challenges in image analysis is robustness. Making all image analysis methods robust enough to use across many cell types and contrast methods is a challenge that needs to be overcome, particularly for non-florescent images. Additional analysis methods and adaptations will be needed to analyzing data in in vivo and 3D environments, which is increasingly common, but difficult to analyze. In addition to havening an extra specially dimension develop the analysis in, z resolution is often poor, which may pose a problem for many analyses. Time resolution may also suffer in these images in order to take a sufficient number of images at varying points in the z dimension. Finally, analyzing imaging through tissues may prove exceedingly difficult due to lack of image clarity and a large number of interfering and interacting cells and debris. Many different things are often in motion in live specimens, causing further challenges in tracking objects across frames.

### 5.4. Acknowledgements

I wish to thank current and former members of the Haugh and Bear laboratories for their contributions these works. These works were supported by grants from the National Science Foundation (#1133476 and CBET-0828936) and the National Institutes of Health grant U54-GM064346.
5.5. References


APPENDIX A*

Detailed Experimental Protocols

A.1 Preparation of Cells and Glass Surfaces for Imaging

The following protocol is used to prepare cells and substrata for TIRF imaging and is intended to aid researchers who are relatively new to live-cell imaging. These steps describe transient transfection of the biosensor into the cells by lipofection, but alternatively one can create stable cell lines by retroviral or lentiviral transfection. If a stable cell line is available, one may skip to step 3. All steps should be performed in a tissue culture biosafety cabinet using proper sterile technique.

Materials

Tissue culture facilities (biosafety cabinet, CO₂ incubator, vacuum aspirator, phase contrast microscope)

35-mm, tissue culture-treated dishes

Dulbecco’s phosphate-buffered saline (PBS)

Fetal bovine serum (FBS)

Appropriate cell culture medium with FBS and antibiotics

DNA plasmid encoding the biosensor

Appropriate transfection reagents

Microcentrifuge tubes

Glass-bottom dishes

Fibronectin, poly D-lysine, or other adhesion-promoting molecule(s) (if needed)

Trypsin-EDTA (if needed)

Centrifuge
**Transfect the cells**

1. Two days before imaging, plate cells in a 35-mm cell culture dish so that they will be 80-90% confluent on the day of imaging. Use standard growth medium.

   In our laboratory, NIH 3T3 cells are transfected when they are 50-70% confluent, one day after plating. In general, the optimum number of cells to plate will depend on the growth rate of one’s cells and the specific transfection protocol used.

2. On the day before imaging, transfect the cells with the biosensor plasmid.

   Transfection protocols will vary depending on the transfection reagents and cells used (UNITS 20.3-20.7, 24.4). For NIH 3T3 cells we typically use Lipofectamine and Plus Reagent from Invitrogen. We dilute 1 μg plasmid DNA (if using 2 plasmids, use 500 ng each and mix them prior to adding any other reagents) in 100 μL of serum-free Opti-MEM medium. To this we add 4 μL of Plus Reagent with gentle mixing. In a separate tube, 2 μL Lipofectamine is diluted in 100 μL Opti-MEM. After incubating the two solutions for 15 minutes at room temperature, they are gently mixed together and incubated for another 15 minutes. During this time, the medium in the cell culture dish is replaced with 1 mL Opti-MEM, preheated to 37°C. Finally, the DNA-
polyplex mixture is added drop-wise to the cells and mixed by gentle swirling. The cells are then returned to the incubator for a 3-hour incubation (the duration of this incubation should be optimized for each cell type). Thereafter, 1.2 mL of cell culture medium containing twice the normal concentration of FBS is added to yield the normal serum concentration.

Prepare the surfaces for cell adhesion

3. Approximately 4 hours before imaging, incubate glass-bottom dishes with 1 mL solution containing an adhesion-promoting protein or polymer for 1 hour at 37°C. The coating solution should be balanced on the glass part of the dish. The volume of coating solution may be adjusted as needed.

   Incubation time and coating concentration should be optimized for each application.

   For NIH 3T3 cells we typically use 10 μg/mL fibronectin or 0.1-1 mg/mL poly-D-lysine, diluted in sterile, deionized water (dH₂O).

4. Carefully tilt the dish and aspirate the excess solution from the edge of the plate. Rinse with sterile dH₂O, taking care not to disturb the coating. Allow the coating to air dry in the hood during the next steps.
Plate the cells

5. Preheat PBS, trypsin/EDTA, and imaging buffer with the desired amount of FBS to 37°C.

*The amount of serum is chosen depending on the duration of the experiment, cell type, and whether or not the cells will be stimulated during the experiment. We typically supplement our usual imaging buffer (see Reagents and Solutions) with 1% FBS to maintain cell viability during experiments lasting several hours. Any cell culture medium lacking phenol red and with added HEPES should also be suitable.*

6. Gently wash cells with ~1 mL Dulbecco’s PBS.

7. If necessary, add 0.5 mL trypsin/EDTA solution to the cells and incubate at 37°C for up to 5 minutes.

*Some cell types may not require trypsin to detach, in which case this step can be skipped. Otherwise, incubate for the minimum time needed to loosen the cells.*

8. Add 1 mL of imaging buffer. Detach and disaggregate cells by pipetting up and down.

Collect the cell suspension in a microcentrifuge tube.

9. Centrifuge the cells 3 min at 300 × g, room temperature.
10. Aspirate the supernatant and resuspend the pellet in 1 mL imaging buffer.

11. Quantify the cell density of the suspension and dilute to a final density of 10,000 cells/mL.

   A hemacytometer or Coulter particle counter with appropriate settings may be used to determine the cell density. The final cell density should be optimized for the particular application. Cells should be sparse enough that most cells will not be touching each other, but one should also be able to find plenty of cells expressing the biosensor at the desired level.

12. Add ~ 1 mL of the cell suspension to the glass part of the dish (~30 cells/mm²) and place in the incubator for a minimum of 2 hours to allow cell spreading.

   Depending on the application and the concentration of serum chosen, one may be inclined to incubate the cells overnight. If so, the protocol would need to be started a day earlier. The longer incubation potentially allows for better acclimation of the cells but might also adversely affect the level of the transiently expressed biosensor. Note too that many cells deposit and/or remodel extracellular matrix proteins on the surface, and thus the surface composition can change significantly with time.
A.2 Live-Cell Imaging Using TIRF Microscopy

This protocol describes a general protocol for live-cell imaging using either an objective- or prism-based TIRF microscope (Toomre, 2012). All of the steps are to be conducted in a dark room/area of the laboratory with dedicated space for the TIRF microscope, image acquisition system, and associated vibration damping and environmental controls. It is expected that practitioners of this protocol will have been trained in laser safety and other relevant safety procedures.

Before using the TIRF microscope, one should confirm that the setup is optimally aligned for near-uniform illumination by each laser. Soluble fluorescent dyes (e.g., Alexa-conjugated dextrans) work well for checking the alignment and adjusting as needed. If desired, images of the illumination field with and without dye can be used to create a mask for shading correction (Wolf et al., 2013).

**Materials**

- TIRF microscope rig with appropriate lasers and filters
- Cooled charge-coupled device (CCD) or complementary metal oxide semiconductor (CMOS) digital camera with appropriate acquisition software
- Immersion oil ($n = 1.52$).
- Humidification system or mineral oil to minimize evaporation
- Heating system for stage/enclosure
- Dye solutions for system optimization/shading correction
Transfected cells plated on coated cover slips or glass-bottom dishes

Prepare the microscope and mount the sample

1. Preheat and humidify the stage chamber at least 1 hour before imaging. Turn on lasers and allow them to stabilize for at least 30 minutes before imaging. If one is also using a mercury lamp for wide-field fluorescence in tandem with TIRF, this should be powered on before the lasers or any other nearby electronics to avoid the risk of a power surge. In lieu of humidifying the chamber, one may be able to layer mineral oil on top of the buffer or otherwise seal the sample to prevent evaporation.

2. Using lens paper and methanol, carefully clean the objective, prism (if used), and the bottom side of the glass-bottomed dish. Our lab uses a prism-based setup with an upright microscope, where the prism is below the sample and the objective dips into the buffer from above. For this reason we are especially vigilant about this cleaning step.

3. Add 1 small drop of immersion oil to the objective lens or center of the prism, position the sample and objective and allow to stabilize for ~15 minutes before imaging. It is critical that no bubbles are present in the immersion oil during this step.
An alternate contrast method may be used at this juncture to identify the correct focal plane before attempting to view the cells under fluorescence. Hence, if the TIRF signal is poor it is likely an issue with the transfection or possibly the laser alignment.

**Set image acquisition parameters**

4. Set the exposure time and gain so that many cells will be clearly visible and select a field to image.

   *Select cells expressing the biosensor at levels that are sufficiently above the background, but not necessarily the very brightest cells. Cells expressing excessive levels of biosensor may show abnormal morphology and reduced motility, in which case they should be systematically avoided. If one intends to perform analysis of individual cells, cells that are touching or very close to other cells should also be avoided.*

5. As needed, readjust the exposure time and gain settings for the selected field to use a high fraction of the camera’s dynamic range. To that end, one should anticipate the magnitudes of expected intensity changes elicited by cell stimulation or drug treatments.

   *For a given laser power, increasing the exposure time boosts the integrated fluorescence intensity but also increases the degree of photobleaching and*
phototoxicity. The exposure time also limits the frequency with which sequential frames can be acquired. If multiple cells in the field are to be analyzed, the settings should be optimized so that the greatest number of cells will be comfortably in the dynamic range of intensity values.

6. Program the acquisition software to acquire images with a time interval and total experiment duration appropriate to capture the dynamic process(es) under study.

   For slow-moving cells like fibroblasts, in which the lifetime of 3’ phosphoinositides is ~ 1 minute, a time interval in the range of 0.5-2 minutes is typically suitable; however, greater temporal resolution may be needed for other cell types or if another, faster process is also being monitored, e.g. by two-color TIRF.

A.3 Critical Parameters

   Important factors in the design of a live-cell microscopy experiment should be considered during sample preparation, image acquisition, and image analysis. In sample preparation, the most important parameters are cell density, transfection efficiency, and biosensor expression level. Cells should be as dense as possible but yet with few cells touching. If too many cells are touching, one will have difficulty separating them in the images (Appendix B.1); there is also the concern that cell behavior will be drastically altered by contact inhibition. As prescribed in Appendix A.1, we find that a density of ~30 cells/mm² is fairly optimal for well-spread cells such as fibroblasts. Quantifying the cell
density of the suspension prior to plating and applying the same, optimal number of cells in each experiment is highly recommended. Maximizing the transfection efficiency is equally important because, for a fixed cell density and magnification, the greatest number of fluorescent cells will be captured in each field of view. The number of useable cells is also affected by the distribution of biosensor expression level in the cell population. We find that there is a good range where the cells are neither too dim nor too bright. For transient expression driven by a strong promoter, we find that the brightest cells are not suitable, as diagnosed by an altered morphology and motility phenotype, which is plausible since the biosensor competes with endogenous phosphoinositide-binding proteins (alternatively, high biosensor expression could foster non-specific cell toxicity). Provided that the biosensor is fairly inert at moderate expression levels, stable integration and selectable expression of the biosensor cDNA is ideal, because all cells in the field will be fluorescent, and the distribution of expression levels tends to be noticeably narrower as compared with transient transfection. Other, more context-specific sample preparation parameters that should be considered and optimized include the surface coating conditions and imaging buffer composition.

Important image acquisition parameters include the laser power density, exposure time, gain setting, exposure interval, and experiment duration. Power density and exposure time go hand in hand, as the product of the two determines the intensity of light that is ultimately detected by the camera. A constraint on the exposure time is that it must be far shorter than the time scales of all processes under study. Unless the process of interest is very fast, a sub-second exposure time (e.g., in the range of 100-500 ms) should be sufficiently short. As for the power, one should weigh the need for good signal to
background against the detriments of long-term photobleaching and phototoxicity. For our prism-based rig, we favor using lasers with higher power (~50 mW) than is minimally needed, but not necessarily to boost signal. Rather, higher power allows us to achieve the same power density with a larger excitation spot, resulting in more uniform illumination. For a given excitation energy, which is usually fixed based on the considerations outlined above, the gain setting should be optimized so that a healthy fraction of the camera’s dynamic range (range of gray levels) are used. To do so, one needs to have developed experience anticipating what changes in intensity might occur during the experimental time course, avoiding saturation of the pixel intensity at all costs. Once the parameters affecting image quality are established, the exposure interval and total duration of the experiment must be optimized. A shorter interval equates to better temporal resolution of rapid processes, whereas a longer overall duration allows one to track slower processes or capture rare events. Together, these parameters will also affect photobleaching of the sample and possibly the strain on local data transfer and storage capabilities.

A.4 Troubleshooting

The principal issue in any live-cell imaging experiment is image quality, which is subverted entirely whenever a TIRF microscope is misaligned. As prescribed under Appendix A.2, a fluorescent dye solution (e.g., a fluorescent dextran in buffer) may be used to assess the uniformity of the illumination. The ideal is a reasonably smooth and flat profile, but lower intensity at the edges of the field can be tolerated if those regions are systematically avoided in the analysis. Irregularities such as blotches or fringes might
indicate an unclean microscope or the presence of bubbles in the immersion oil. One might also use this test to quantitatively compare against archived images using the same dye standard; a buffer only sample should also be run for the purpose of background subtraction. Misalignment can also affect whether the instrument is achieving proper evanescent illumination. The same soluble dye, diluted in a proper imaging buffer, may be added to a sample of adhered cells (either non-fluorescent or expressing a construct that does not clash spectrally with the external dye). If TIRF is being achieved, the cell membrane and cytoplasm above will occlude a fraction of the external dye from the evanescent wave, and therefore the contact zone will appear noticeably dimmer than the rest of the field. As a quantitative example, consider an estimated penetration depth $d = 100$ nm and a characteristic surface to membrane separation $h = 50$ nm. The expected ratio of the contact area intensity ($F_{\text{cell}}$) versus the external intensity ($F_{\text{external}}$) in the dye channel is calculated as follows.

$$\frac{F_{\text{cell}}}{F_{\text{external}}} = 1 - e^{-h/d} \approx 0.4$$

An even stronger test is achieved when the dye control is run with cells expressing an intracellular volume marker, viewed in a separate channel. Under proper TIRF conditions, the subtle variations in fluorescence intensity should mirror each other. That is, wherever the external dye channel shows a bit lower intensity within the contact area (interpreted as a lower $h(x,y)$), the cell volume marker should show a correspondingly higher intensity. Another hallmark of a true TIRF image is its crispness. If the microscope is properly focused, the cell contact area should show noticeably sharper edges than those acquired by
other fluorescence microscopy modes (for which z-resolution is the proverbial Achilles’ heel). By the same token, TIRF is sensitive to the focus. To focus the microscope near (but not necessarily spot on) the optimum focal plane, we recommend using a transmitted light modality before switching to TIRF.

A.5 References


APPENDIX B*

Detailed Analysis Protocols

B.1 Basic Image Processing and Segmentation

For most analyses, images must be processed to identify and group those pixels comprising the projected area of each cell (Fig 4.1). With fluorescence images, this step may be performed by binarizing the image based on a threshold intensity value. In this protocol and those hereafter, only computational resources are required (a computer workstation running MATLAB and the freeware ImageJ), and therefore no materials are listed. One should have first acquired a working knowledge of programming in MATLAB before attempting this protocol or those remaining.

**Pre-process the images using ImageJ**


2. Open the image stack or sequence in ImageJ: File→Open or File→Import→Image Sequence.

   *If one’s images are of a file type not recognized by ImageJ, a possible fix is to install an ImageJ plugin such as the Bio-Formats library. If this does not work, one might need to convert the images to a more general format using the native software. If so, one should avoid compression and preserve bit depth whenever possible.*

3. Crop a single cell out of the image by selecting the area on the image with the rectangular selection tool and then: Image→Crop Image (see Fig. 1a&b).
Make sure the rectangular selection is large enough that the cell remains in the region for the duration of the movie. If images were acquired in more than one channel, the selection can be saved using the ROI manager before cropping the cell, and thus the same area may be cropped in the other channel. If another cell enters the region during the movie, one can remove it by selecting a region around it and setting those pixels to zero or the background level: Process→Math→Set.

Alternatively, one can deal with such cells in MATLAB later. The MATLAB function ‘imclearborder’ will remove structures that are touching the edge of the cropped image.

4. Scale and convert the image to grayscale if necessary.

If one’s images are in RGB, they should be converted to grayscale:

Image→Type→The current bit depth of the image (probably 8-bit). With either grayscale or RGB images, if one must convert to a lower bit depth to save memory, first rescale the image using: Image→Adjust→Brightness/Contrast, so that as much of the dynamic range is used as possible.

5. Save Images as sequential tiffs: File→Save As→Image Sequence.
Give each cell a unique file name identifier or save each image sequence in a separate directory. To simplify the next few steps, we recommend creating a folder to save them in the MATLAB directory.

**Threshold the image and create masks using MATLAB**


   Alternatively, one can create a function file that is called each time a cell is to be processed. It is good practice to add a few comment lines at the beginning of the file to explain its purpose and use. Comments are added by placing the ‘%’ character before the text.

7. Save the M-File: File → Save As.

   If the file is saved in a different directory than the images, one will need to add the path of the file, or MATLAB will prompt for it. One can add the path using the ‘addpath’ function.

8. In the program, declare the file name, file folder, and directory as variables.

   If one has sequentially numbered images, an asterisk in the filename may be used as a wildcard. For example, suppose that one has sequential images starting with
‘AKT0000’ in a folder called ‘4-25-11 cell 5’ which in turn is contained in a folder called ‘work’ in the MATLAB root directory. The variable declarations would look like this:

```matlab
FolderNames='4-25-11 cell 5';
filename='AKT*.tif';
directory = fullfile(matlabroot,'work',FolderNames);
```

9. Change to that directory using ‘cd’ and read the file names using ‘dir’ as follows.

```matlab
cd(directory)
dirOutput = dir(fullfile(directory, filename));
fileNames = {dirOutput.name};
```

10. Count the number of frames and initialize the matrix to store the cell masks as follows.

```matlab
numFrames = numel(fileNames); %Count the number frames
I = imread(fileNames{1}); %Load the first image to get the class and size
cellmask = zeros([size(I) numFrames],class(I)); %Initialize the matrix for the masks
```
If a matrix changes in size during a loop, the matrix should be initialized to its largest size first. This step avoids the need to recreate the matrix each time, which is more resource intensive.

11. Create a loop to read the images that will be thresholded.

Depending on the size and number of images and the available RAM, one may want to save these to a variable to avoid having to reread them multiple times.

12. Determine the level of the image threshold.

This can be done several ways, such as averaging the intensity of a background region and choosing a value somewhat higher than that or performing a line scan across the cell boundary and using a value near the point of inflection. Using a threshold value a certain percentage above the background level is typically more reliable than an absolute offset, which is prone to errors from artificial intensity changes, such as fluctuations in laser power. Alternatively, the lowest bin from k-means segmentation may be used to identify the background (see below).

13. Finally, create a binarized cell mask using the function ‘im2bw’ and the previously identified thresholding level.
B.2 Segmentation of PI3K Signaling Hot Spots

We have previously reported that recruitment of FP-Akt-PH biosensors is enriched in protruding lamellipodia of migrating fibroblasts, localized in regions that we refer to as PI3K ‘hot spots’ (Melvin et al., 2011; Weiger et al., 2010). Identification of these regions by segmentation (Fig. 4.1c) enables several forms of analysis that can provide further insight into spatiotemporal dynamics of PI3K signaling (or of any other, intensity-based readout). This process is straightforward if the images have already been processed according to Appendix B.1.

Identify and group high-intensity pixels using MATLAB

1. Prepare and load images into MATLAB as described under Appendix B.1.

   If desired, one can obtain an intensity image of the cell with no background by multiplying the previously obtained cell mask by the original, background-subtracted image. The k-means clustering method to be used in subsequent steps should also segment the cell from the background.

2. Determine areas of locally increased signaling by k-means clustering. We use a function available here:

Rename this function (‘kmeans1’ below) to distinguish it from the native MATLAB function.

\[
[\sim, c1] = \text{kmeans1}(I, k);
\]

The optimum number of bins, ‘k’, will need to be determined empirically. In past work we have used \(k = 4\); the highest intensity pixels, including those grouped as hot spot regions, are found in bin 4, whereas the lowest intensity pixels in bin 1 are associated with the cell background. As an alternate (and sometimes more robust) method, one can first identify the cell-associated pixels and then distinguish the high-intensity pixels as those with intensity values exceeding the mean by a certain multiple of the standard deviation.

3. Designate the highest intensity bin as hot spot pixels. In the code below, the associated mask is named ‘hotspotim’.

\[
[a, b] = \text{size}(I);
\]
\[
\text{hotspotim} = \text{zeros}(a, b);
\]
\[
\text{for } j=1:a
\]
\[
\quad \text{for } z=1:b
\]
\[
\qquad \text{if } c1(j, z)==k
\]
\[
\quad \quad \text{hotspotim}(j, z)=1;
\]
\[
\quad \end
\]
4. We recommend filtering contiguous hot spot regions using a certain minimum pixel size threshold. This can be done using the function ‘bwareaopen’.

\[
\text{hotspotim} = \text{bwareaopen(hotspotim, minsize)}; \quad \% \text{remove hot spots smaller than minsize}
\]

In choosing the size threshold, one should consider the nature of the analyses that will be performed on the hot spot regions as well as the spatial resolution and clarity of the images.

B.3 Calculating the Macroscopic PI3K Signaling Vector

Having established basic protocols for experimental preparation, image acquisition, and image processing, the following protocols outline some more advanced methods for analyzing the spatiotemporal dynamics of PI3K signaling (and, more generally, of any intensity-based readout) as it relates to cell morphology and motility (Fig. 4.2a). The first of these advanced protocols outlines a method to represent the overall orientation of PI3K signaling hot spots as a vector. The *macroscopic signaling vector* is a weighted sum of the vectors drawn from the centroid of the cell contact area to those of the aforementioned
signaling hot spot regions identified by segmentation in Appendix B.2 (Weiger et al., 2009, 2010). Having both a direction and a magnitude, the vector is an appropriate metric representing the overall asymmetry/anisotropy of the biosensor translocation pattern. We have demonstrated how dynamics of the signaling vector may be analyzed, manifest as a wind rose plot (Weiger et al., 2009), as a correlation with the cell centroid displacement vector (Weiger et al., 2010) (Fig. 4.2a), and as a path with successive signaling vectors joined head to tail (Melvin et al., 2011).

**Determine the vector from hot spot characteristics using MATLAB**

1. Perform image segmentation as prescribed by Appendix B.2. The hot spot mask is named ‘hotspotim’.

2. For each cropped image, determine the centroid, area, and average intensity of each hot spot and the centroid of the cell using ‘regionprops’. A labeled image of each hot spot can be obtained using the function ‘bwlabel’, which can be used to call ‘regionprops’.

```matlab
labelspots=bwlabel(hotspotim);  
STATSpots=regionprops(labelspots,spotsintensity,'Area','Centroid','MeanIntensity'); %spotsintensity is an intensity image of the just the hot spots  
labelcell=bwlabel(cellmask); %cellmask is a binary image of the cell  
STATSCell=regionprops(labelcell,'Area','Centroid');
```
2. Check for multiple cell fragments and use the largest one for the centroid.

OGSize=cell2mat({STATS.Area});
[OGSortSize index]=sort(OGSize,'descend');
AllCenter=cell2mat({STATS.Centroid});
Center(1)=AllCenter(2*index(1)-1);  %Centroid x coordinate
Center(2)=AllCenter(2*index(1));    %Centroid y coordinate

3. For each hot spot, determine the $x$- and $y$-components of its position vector relative to the cell centroid (‘vXs’ and ‘vYs’, respectively). Then, rescale this vector so that its magnitude is equal to the fluorescence volume (mean intensity*area). The rescaled components of the vector are ‘vXcomp’ and ‘vYcomp’.

vXs=zeros(length(STATSspots,1));
vYs=zeros(length(STATSspots,1));
vXcomp=zeros(length(STATSspots,1));
vYcomp=zeros(length(STATSspots,1));
for i=length(STATSspots)
    vXs(i)=STATSspots(i).Centroid(1) - STATScell.Centroid(1);
    vYs(i)=STATSspots(i).Centroid(2) - STATScell.Centroid(2);
    vmag=STATSspots(i).MeanIntensity * STATSspots(i).Area;
    dist=sqrt(vXs(i)^2+vYs(i)^2)
    vmagnorm=vmag/dist;
    vXcomp(i)= vXs(i)*vmagnorm;
    vYcomp(i)= vXs(i)*vmagnorm;
4. Sum the individual signaling vectors to calculate the macroscopic signaling vector for the current image.

\[
totX = \text{sum(vXcomp)}; \\
totY = \text{sum(vYcomp)};
\]

5. Repeat these calculations in a loop for each frame, saving the desired variables to a matrix.

6. If desired, correlate the macroscopic signaling vector with other vectors of interest, such as that of cell movement. We have used the vector correlation coefficient as defined in Hansen et al. (Brian Hanson, 1992).

*The vector of cell movement is determined by subtracting the cell centroid coordinates in successive frames/time intervals. A time interval large enough for significant cell centroid movement should be used. To correlate only the orientations of the two vectors, rescale their magnitudes to make them unit vectors.*
B.4 PI3K Hot Spot Tracking

The previous protocol outlined the construction of a signaling vector from intensity data contained in a single image. Although the method is relatively simple to implement, one can only infer dynamical information from it by assessing changes in the signaling vector as a function of time. In the following protocol, we prescribe a method for tracking individual hot spots and determining their fates (Weiger et al., 2010) (Fig. 4.2b). It uses the same data already curated to implement Appendix B.3. The algorithm starts with a hot spot region in the previous image and checks for spatial overlap with a hot spot, or multiple hot spots, in the next frame(s). If only one such hot spot is found, it is identified as the same hot spot as in the previous image. If none is found, then the hot spot is declared dead. If instead two (or more) hot spots are found, the “new” hot spots are said to have been born by branching and are linked historically to their progenitor. Carrying out this operation in reverse identifies instances when two (or more) hot spots merge into one. Finally, any hot spot not yet accounted for is considered to have been born spontaneously.

1. Calculate hot spot properties (label structures, centroid coordinates, mean intensity, and area) for all frames, to be used later. Initialize the matrices using a conservative estimate of the maximum number of hot spots, ‘totalhs’.
Assuming that the first frame has a typical number of hot spots, a conservative estimate of ‘totalhs’ is the number of hot spots found in the first frame times the number of frames.

%Initialize matrices
labelspots=zeros([size(hotspotim) numframes]);
Xs=zeros(totalhs,numframes);
Ys=zeros(totalhs,numframes);
mag=zeros(totalhs,numframes);
areas=zeros(totalhs,numframes);

for f=1:numframes
    hotspotim=imread((fileNames{f}));
    labelspots(:,:,f)=bwlabel(hotspotim);
    spotsintensity=imread((fileNames2{f})).*hotspotim;
    STATSspots=regionprops(labelspots(:,:,f),spotsintensity,'Area',
                            'Centroid','MeanIntensity');
    for i=1:length(STATSspots)
        cx=STATSspots(i).Centroid(1);
        cy=STATSspots(i).Centroid(2);
        Xs(i,f)=cx;
        Ys(i,f)=cy;
        mag(i,f)= STATSspots(i).MeanIntensity ;
        areas(i,f)= STATSspots(i).Area;
    end
end
2. Create a master matrix (‘mastersav’) for hot spot properties that will be tracked: centroid, area, intensity, and fate. Along with the aforementioned ‘totalhs’, provide a conservative bound on the maximum number of hot spots that will be found in consecutive frames (‘maxtracked’).

For maxtracked, a conservative bound is ~3 times the number of hot spots in the first frame.

mastersav=zeros(numproperties,totalhs,numframes);
indexused=zeros(maxtracked,numframes);
cursp=0; %Initialize the current Spot identifier being tracked

3. Create a nested loop structure with a master ‘if’ statement. The intervening codes (‘Area 1 code’, etc.) will be covered in the steps that follow.

for f=1:numframes %Outer time loop
    %Area 1 code here
    for i=1:max(max(labelspots(:,:,f))) %Hot spot loop
        %Area 2 code here
        if skip==0; %Skips hot spots that have already been tracked
            %Area 3 code here
            while death==0 && frame<=numframes-1 %Inner time loop
                %Area 4 code here
            end
        end
    end
end
In the inner time loop, each hot spot is tracked until death. The hot spot loop cycles through all of the hot spots. Once all hot spots present in the first image have been tracked, the outer time loop moves on to the next frame, and the procedure is repeated. The ‘if’ statement determines whether or not a hot spot has previously been accounted for. If so, that hot spot is skipped.

4. In ‘Area 1 code’, initialize the index counter, ‘indexcount’. This is to keep track of the location of the current ‘i’ value of the spot within the matrix ‘indexused’ as the spot is being tracked in time.

    indexcount=0;

5. In ‘Area 2 code’, set ‘skip’ equal to zero initially, then switch ‘skip’ to 1 if the current spot has already been tracked.

    skip=0;
    for ckin=1:length(indexused(:,1));
        if i==indexused(ckin,1);
            skip=1;
    
end
end
end
6. In ‘Area 3 code’, add one to the current hot spot identifier (‘cursp’), initialize the ‘death’ variable, and set the starting frame for the inner time loop equal to the current position of the outer time loop. Thus, only frames after the birth of the hot spot will be checked. The code that follows stores the data for each new (previously untracked) hot spot.

```matlab
cursp=cursp+1;  %Save the next hot spot data in a new column
death=0;
frame=f;

mastersav(1,cursp,frame)=Xs(i,frame);
mastersav(2,cursp,frame)=Ys(i,frame);
mastersav(3,cursp,frame)=areas(i,frame);
mastersav(4,cursp,frame)=mag(i,frame);
mastersav(5,cursp,f)=1;
```

*Hot spot property 5 is the hot spot fate, which will be updated in each successive frame. The new hot spot is assigned a fate of 1 (birth). Note that, in any analysis of fate statistics performed later, births in the first frame should be excluded.*

7. In ‘Area 4 code’, create a matrix (‘imt’) to serve as a mask of the current hot spot pixels.
if frame==f; %If the spot was just born
    [R,C]=find(labelspots(:,:,frame)==i);
else %If the spot is already being tracked
    curi=indexused(indexcount,frame);
    [R,C]=find(labelspots(:,:,frame)==curi);
end
imt=zeros(size(labelspots(:,:,1)));%imt contains only the current spot
for yy=1:length(R)
    imt(R(yy),C(yy))=1; %imt contains only the current spot
end

8. Continuing in the ‘Area 4 code’, identify the spots in future frames that overlap the
current hot spot mask and their hot spot loop numbers. Hence, tracking will continue
unless no overlap is found.

overlap=imt & labelspots(:,:,frame+1); %Calculate overlapping areas
[R,C]=find(overlap);
lck=zeros(size(overlap));
for yy=1:length(R)
    lck(R(yy),C(yy))=labelspots(R(yy),C(yy),frame+1);
end
labelnums=unique(lck); %Pick out the hot spot loop numbers

*It might be prudent to check for overlap in the ‘frame+2’ frame (or more) also. This
gives one the option of tracking a hot spot that was “lost in segmentation” for one*
frame. One might also choose to disregard hot spots that were present in only one frame.

9. Continuing in the ‘Area 4 code’, identify hot spot fates using the hot spot loop numbers. If no loop numbers are found, the hot spot has died (fate = 2). If one is found then the hot spot has survived (fate = 5). If multiple loop numbers are found, the hot spot has branched (fate = 3). Merging events (fate = 4) are handled in the next step.

```matlab
if (max(labelnums))>0
    if length(labelnums)>2
        mastersav(5,cursp,frame)=3; %Branching
    else
        if frame~=f
            mastersav(5,cursp,frame)=5; %Survival
        end
    end
else
    mastersav(5,cursp,frame)=2; %Death
    death=1;
end
```

As with the previous step, it may be useful to check more than one frame into the future. This gives one the option of disregarding spurious branching and merging events.
10. At the end of the ‘Area 4 code’, record the data for surviving hot spots. Before progressing to the next frame, check for hot spot merge events. If a surviving hot spot has the same centroid as a previously tracked hot spot, mark it as a merge event (fate=4).

```matlab
if death==0
    zlnum=nonzeros(labelnums);
    indexcount=indexcount+1;
    indexused(indexcount,frame+1)=zlnum(1);
    mastersav(1,cursp,frame+1)=Xs(zlnum(1),frame+1);
    mastersav(2,cursp,frame+1)=Ys(zlnum(1),frame+1);
    mastersav(3,cursp,frame+1)=areas(zlnum(1),frame+1);
    mastersav(4,cursp,frame+1)=mag(zlnum(1),frame+1);
    xallspot=mastersav(1,:,frame+1);
    yallspot=mastersav(2,:,frame+1);
    for xss=1:length(xallspot)
        if cursp ~= xss
            if xallspot(xss)==mastersav(1,cursp,frame+1) &&
                yallspot(xss)==mastersav(2,cursp,frame+1)
                mastersav(5,cursp,frame)=4;
            end
        end
    end
end
frame=frame+1;
```
B.5 Constructing a Spatiotemporal map of PI3K Signaling

Here we outline a method to visualize the dynamics of PI3K signaling, manifest as AktPH translocation monitored by TIRF microscopy, with respect to location and time (Welf et al., 2012) (Fig. 4.2c). This approach is similar to kymograph-like methods used to visualize cell motility dynamics (Machacek and Danuser, 2006), with protrusion/retraction velocity replaced with intensity information.

**Bin the intensity data according to angular position relative to the cell centroid**

1. Segment the PI 3-kinase hot spots as described under Appendix B.2.

   As an alternative, or in combination with hot spot segmentation, one can restrict the analysis to the cell periphery, found by eroding the cell boundary (MATLAB function ‘imerode’) and subtracting the resulting image from the cell mask. If this method is used without segmentation, normalization of the signaling map (Step 7) is advised.

2. Create a hot spot intensity image by multiplying the background-subtracted image by the hot spot mask.

   \[ HS_{int} = \text{Intensity} \times HSmask; \]
Be sure to use `.*` instead of `*` to multiply the individual elements of the matrix rather than doing matrix multiplication.

3. Determine the cell centroid and area using `regionprops`.

```matlab
labelcell = bwlabel(cellmask);
STATS = regionprops(labelcell, 'Centroid', 'Area');
```

4. As in Appendix B.3, when there are multiple cell fragments, use the largest one for the centroid.

```matlab
OGSize = cell2mat({STATS.Area});
[OGSortSize index] = sort(OGSize, 'descend');
AllCenter = cell2mat({STATS.Centroid});
Center(1) = AllCenter(2*index(1) - 1);  % Centroid x coordinate
Center(2) = AllCenter(2*index(1));    % Centroid y coordinate
```

5. In a loop for each image (indexed as ‘frame’), use the ‘find’ function to locate the coordinates of all hot spot pixels.

```matlab
[SpotY, SpotX] = find(HS(:,:,frame));
```
6. In the same loop, find the angle of each pixel relative to the cell centroid and sum the intensities within each angle bin. ‘HSalpha’ stores the angle of the current pixel, while ‘HSLog’ stores the sum of the intensities in each bin.

\[ HSalpha = [] ; \]
\[
\text{for } i = 1: \text{length(SpotX)} \% \text{for each hot spot pixel}
\]
\[
\text{HSalpha} = \text{atan2}((\text{SpotY}(i) - \text{Center}(2)), (\text{SpotX}(i) - \text{Center}(1))) \times 180 / \pi + 181 ;
\]
\[
\text{HSLog}(k, \text{fix(HSalpha)}) = \text{HSLog}(k, \text{fix(HSalpha)}) + \text{intensity(SpotY}(i), \text{SpotX}(i));
\]
\text{end}

*Be sure to initialize HSLog before the loop as follows.*

\[
\text{HSLog} = \text{zeros(numframes, angles)} ;
\]

7. If desired, normalize the value in each bin by the total number of pixels in the bin.

*Depending on how step 1 is performed, normalization may be necessary to avoid artifacts resulting from irregularities in cell shape, such as elongated structures.*

8. If desired, smooth the resulting matrix (MATLAB function ‘smooth’).
Depending on the image resolution, temporal resolution, and angle bin size it may be necessary to smooth the matrix either spatially or temporally or both.

9. Display the matrix as an image and apply an appropriate colormap as follows.

```matlab
imagesc(HSLog)
colormap('default')
```

One can adjust the colormap using the command ‘colormapeditor’.

10. If desired, produce a protrusion/retraction map as well. Protruded pixels (‘Prots’) are those that are cell-associated in the current frame but not in the previous frame; retracted pixels (‘Rets’) are those that were cell-associated in the previous frame but not in the current frame.

```matlab
change=Pres-Prev; %negative values show retraction; positive protrusion
Prots(find(change>0))=1;
Rets(find(change<0))=1;
```

After determining the protrusion and retraction masks, go to step 6 using these pixel coordinates instead.
11. If desired, correlate the signaling and protrusion (or any two) maps.

   *This may be done with a time lag or spatial offset using the MATLAB function*

   ‘normxcorr2’ *on the two matrices.*

   ```matlab
corr=normxcorr2(HSLog,PLog);
```

   *To correlate with a time lag only (no spatial offset), use the following to call that data from the ‘corr’ matrix.*

---

**B.5 References**


