Cellular morphodynamics are critically important in a variety of normal and pathological processes, including neuronal cell migration in development, leukocyte transendothelial migration (TEM), and cancer cell metastasis. The Rho small GTPases function as molecular switches that transduce a variety of stimuli into cellular morphodynamic changes. The importance of these molecules in aberrant cell migration is evident in the large body of work demonstrating that GTPase overexpression is prevalent in a variety of human cancers and is associated with poorer prognoses. In addition, mutations have been found in many upstream regulators of Rho GTPases in human cancers, leading to overactivation of these GTPases, tumor formation, and metastasis. Similar genetic and molecular changes can be found in other diseases as well, including a variety of inflammatory diseases. Rac1, RhoA, Cdc42, and RhoG are key Rho GTPases that work together to regulate cell morphology. Biochemical data suggest that RhoG and Cdc42 can activate Rac1 through several mechanisms, and that Rac1 and RhoA are mutually inhibitory. These relationships may play important roles in the proper orchestration of cell migration events, with aberrancies occurring during pathological disease processes. Testing these hypotheses requires measurement of Rho GTPase activation and deactivation kinetics in real time, with single microns and seconds resolution. Such measurements are best obtained through activity biosensors, since GTPase localization does not correlate well with GTPase activity.

To probe these relationships between the Rho GTPases during cell migration, I divided my research goals into three major aims:

1) Design, improve, and compare new Forster Resonance Energy Transfer (FRET)-based biosensors for Rac1, RhoA, Cdc42, and RhoG to permit access to a greater variety of Rho GTPases with greater sensitivity, along with the ability to image two or more GTPases simultaneously within living cells and *in vivo*.
2) Determine whether Rac1, RhoA, Cdc42, and RhoG activities are coordinately regulated within migrating cells, and whether their behaviors fit the proposed model whereby RhoG, Rac1 and Cdc42 are active at the leading edge, with RhoA active primarily within the retracting tail of cells.
3) Determine the spatial and temporal mechanisms by which RhoG, a lesser known GTPase, regulates Rac1 activity within migrating cells.

Toward the first aim of my dissertation proposal, I have developed new sensor designs for RhoA, Rac1, RhoG, and Cdc42 through significant optimization and characterization of intermolecular FRET designs. Each of these new sensors attains a much greater dynamic range than previous sensors, meaning that they are capable of detecting much lower levels of signaling events within cells, a significant advantage over currently available designs that utilize an intramolecular FRET approach. I have also translated these results into visualizing two or more sensors simultaneously within the same cell, and am working toward their use in living organisms. To accomplish this, I have worked to identify and test new red-shifted fluorescent protein FRET pairs that work well with our currently existing sensors, along with novel delivery methods for our intermolecular FRET sensors. Through many rounds of testing, I have found that monomeric Kusabira Orange pairs well with mCherry as a red-shifted donor/acceptor pair. To date, I have used this new FRET pair in our RhoA and Rac1 sensors to detect RhoA and Rac1 activities, respectively. My work has yielded a variety of new sensors, sensor designs, and orthogonal FRET sensors that greatly expand our capacity to study Rho GTPases within living cells and organisms. One additional hurdle I have sought to overcome is the delivery of these novel sensors to a variety of cell types from a single construct with controlled expression levels of the two components from a single plasmid. By incorporating a self-cleaving amino acid sequence from the Hepatitis Delta virus in these new FRET sensors, I have been able to achieve excellent control over chain stoichiometry, previously a major barrier. I have incorporated these constructs into adenoviral vectors for expression of my sensors in nearly any cell line or tissue available. I am finishing the final experiments in this aim, studying how introduction of these sensors at various concentrations within cells affects downstream signals, and comparing different sensor designs to determine which design is more sensitive to changes in cellular signaling. My work here will result in a first-author publication targeted to either *Nature Methods* or *the Biophysical Journal*.

Arising from my design work above, I have spearheaded a number of collaborations where I have used these new sensors to answer questions regarding the regulation of Rho GTPases in cell signaling. In each case, I have proposed new questions and experiments, even designing several new sensors specifically for each experimental question. For example, in my work recently published in the *Journal of Cell Biology* I found that I

needed a Cdc42 sensor with a very high level of FRET in the “on” state, but not low FRET in the “off” state, to better measure Cdc42 activity after EGF stimulation. I thus redesigned the sensor and performed experiments that provided novel insights for the paper inaccessible using our collaborator’s biochemical techniques.

Toward the second aim of my research proposal, I published a co-first-author paper in *Nature* describing the activation patterns of the Rho GTPases RhoA, Rac1, and Cdc42, and their correlation both spatially and temporally with the velocity of lamellipodial protrusion in migratory cells. In this first use of correlation analysis and GTPase activity imaging data I and the other co-authors were able to demonstrate that RhoA was located spatially at the very leading edge, and was activated at the initiation of protrusion, whereas Cdc42 and Rac1 were located spatially distal from the leading edge with a time delay after the initiation of protrusion. I also utilized my work from Aim 1, permitting me to compare different sensor designs at different expression levels in identical cellular behaviors, showing that neither sensor design or expression level influences the correlation analyses, allowing activity patterns generated by different sensor designs to be directly compared. Our results provide a much more complex model of Rho GTPase regulation of cell migration, revealed only by this novel methodology, showing that all three GTPases are coordinated at the leading edge for cell movement, contrasting with previous models from biochemical data alone. Thus, our new model lays the groundwork for a variety of new hypotheses about cytoskeletal regulation that I and others are currently testing in the lab.

My third aim has been to delve more deeply into the biological properties of two interacting Rho GTPases, Rac1 and RhoG. Using the RhoG biosensor I developed in Aim 1, I have explored the role of RhoG in cell migration and its ability to regulate specific subsets of Rac1 activity within migrating cells. In my analysis, I showed that RhoG is activated in migrating cells coincident with protrusion and predominantly at the very leading edge of cells, unexpectedly differing from Rac1, its close homologue which demonstrates both a temporal and spatial lag after protrusion. I also show that knockdown of RhoG expression by siRNA diminishes Rac1 activity within the first 6 μm of the leading edge, but maintains it elsewhere within the cell. I am able to show that these differences are phenocopied by disruption of the ELMO/DOCK180 complex, known to mediate Rac1 activation by RhoG. These changes in activity lead to defects in random motility of HeLa cells through alterations in focal adhesion dynamics, which are mediated by a loss of Rac1 at adhesions. Interestingly, RhoG regulation of Rac1 is mediated by the proper trafficking and localization of RhoG to specific cellular compartments. First, RhoG localizes to recycling endosomes, and reports have identified RhoGDI-3 as a potential regulator of RhoG trafficking to and from the cell membrane. By knocking down RhoGDI-3, I have increased the trafficking of RhoG to and from the membrane, increasing RhoG activity, and aberrantly increasing Rac1 activity, while overexpression of RhoGDI-3 induces the opposite results. The data led me to develop a model in which RhoG spatiotemporally regulates Rac1 activity within the lamellipodium, and are consistent with previous reports suggesting that endosomal trafficking is critical for appropriate spatial regulation of Rac1 activity, pointing to RhoG as a key endosomally-trafficked regulator of Rac1. I am now testing whether manipulation of RhoG in a variety of cancer cell lines diminishes their migratory capacity and/or alters their behavior in 3-dimensional matrices. I intend to submit these results within the next few months.

Lastly, over the next year as I complete my graduate training, I anticipate tackling one final project: the study of Rho GTPase activation during leukocyte transendothelial migration (TEM) in inflammation. The biosensor characterization and design efforts from Aim 1 have given me tools to study how white blood cells extravasate out of the bloodstream and into tissues, particularly in regard to how the vessel endothelium changes its cytoskeletal dynamics to allow white blood cells to escape. This process is critically regulated by Rho GTPases, and thus, over the next year, I intend to study how the GTPases regulate this process. I have already analyzed Rac1 activation downstream of ICAM-1 engagement in COS-7 cells using 3 μm beads coated with anti-ICAM-1 antibody as a simplified test model of TEM. Using a Rac1 biosensor, I visualized and quantified Rac activation downstream of ICAM-1 engagement and clustering, showing that a majority of cells respond to bead adhesion with a transient burst of Rac1 activity compared to controls. I have also begun to extend this analysis to RhoA, Cdc42, and RhoG, since I have tested all the viral constructs needed to perform these studies in primary endothelial cells, and my initial studies demonstrate that I am able to introduce these sensors into endothelial cells at appropriate levels for live-cell imaging, with my analysis of TEM ongoing. Once this analysis is complete, I will begin to perturb the different GTPases to assess how each GTPase contributes to the different stages of leukocyte transendothelial migration, revealing potential new targets for therapeutic intervention in a variety of inflammatory diseases.