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In vivo Analysis and Modeling Reveals that Transient Interactions of Myosin XI, its Cargo, and Filamentous Actin Overcome Diffusion Limitations to Sustain Polarized Cell Growth

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In vivo Analysis and Modeling Reveals that Transient Interactions of Myosin XI, its Cargo, and Filamentous Actin Overcome Diffusion Limitations to Sustain Polarized Cell Growth

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Abstract

Tip growth is a ubiquitous process throughout the plant kingdom in which a single cell elongates in one direction in a self-similar manner. To sustain tip growth in plants, the cell must regulate the extensibility of the wall to promote growth and avoid turgor-induced rupture. This process is heavily dependent on the cytoskeleton, which is thought to coordinate the delivery and recycling of vesicles containing cell wall materials at the cell tip. Although significant work has been done to elucidate the various molecular players in this process, there remains a need for a more mechanistic understanding of the cytoskeleton’s role in tip growth. For this reason, specific emphasis should be placed on understanding the dynamics of the cytoskeleton, its associated motors, and their cargo.

Since the advent of fluorescence fusion technology, various quantitative fluorescence dynamics techniques have emerged. Among the most prominent of these techniques is fluorescence recovery after photobleaching (FRAP). Despite its prominence, it is unclear how to interpret fluorescence recoveries in confined cellular geometries such as tip-growing cells. Here we developed a digital confocal microscope simulation of FRAP in tip-growing cells. With this simulation, we determined that fluorescence recoveries are significantly influenced by cell boundaries.

With this FRAP simulation, we then measured the diffusion of VAMP72-labeled vesicles in the moss Physcomitrella patens. Using finite element modeling of polarized cell growth, and the measured VAMP72-labeled vesicle diffusion
coefficient, we were able to show that diffusion alone cannot support the required transport of wall materials to the cell tip. This indicates that an actin-based active transport system is necessary for vesicle clustering at the cell tip to support growth. This provides one essential function of the actin cytoskeleton in polarized cell growth.

After establishing the requirement for actin-based transport, we then sought to characterize the *in vivo* binding interactions of myosin XI, vesicles, and filamentous actin. Particle tracking evidence from *P. patens* protoplasts suggests that myosin XI and VAMP72-labeled vesicles exhibit fast transient interactions. Hidden Markov modeling of particle tracking indicates that myosin XI and VAMP72-labeled vesicles move along actin filaments in short-lived linear trajectories. These fast transient interactions may be necessary to achieve the rapid dynamics of the apical actin, important for growth. This work advances the field’s understanding of fluorescence dynamics, elucidates a necessary function of the actin cytoskeleton, and provides insight into how the components of the cytoskeleton interact *in vivo*. 
1 Introduction: On Modeling and Parameter Estimation of Plant Polarized Cell Growth

1.1 Introduction
The food and agriculture organization of the United Nations estimates that global crop yields will have to increase by over 50% by the year 2050 to meet the needs of a growing population (BibeeuAlexandratos, 2012). Since agronomic traits are the result of a complex feedback between genetics and environmental stimuli, there is a growing need to better understand these systems fundamentally. One potential avenue is to establish an understanding of the mechanisms associated with plant growth at the cellular level.

A specialized type of plant cell growth is polarized cell growth, in which a cell expands in a unidirectional self-similar manner. Polarized cell growth is important for nutrient uptake by root hairs, for fertilization by pollen, and for gametophyte development by the protonemata of bryophytes and ferns. Because of the presence of the cell wall, the plant cell is under tremendous turgor pressure that is uniformly distributed across the cell wall (Benkert et al., 1997). To promote tip growth, the cell must regulate the material properties of the cell wall, such that the cell tip maintains an extensibility that supports expansion. Furthermore, it must also ensure that the cell wall distal to the tip remains rigid to prevent rupture and keep its long tubular shape. To spatially control the material properties of the wall, the cell performs a combination of directed secretion and endocytosis. Both endocytosis and exocytosis are thought to be dynamically regulated by the cytoskeleton.
Here, we review the existing literature about the mechanism behind polarized growth including wall mechanics, endocytosis, exocytosis, the cytoskeleton, and calcium signaling. We place particular emphasis on the current state of mathematical modeling within this field and describe how recent developments in fluorescence dynamics assays hold great promise in advancing the current understanding of the role of the cytoskeleton in tip growth, by enabling the measurement of essential modeling parameters.

1.2 Model Systems

1.1.1 Pollen Tubes
The best-studied model for tip growth is the pollen tube (Rounds and Bezanilla, 2013). Pollen tube growth serves the primary purpose of delivering the sperm cell to the ovule during sexual reproduction. Pollen tubes are approximately 16 $\mu$m wide and can grow up to a foot long in maize (Zea mays) (Barnabas and Fridvalszky, 1984). Compared to root hairs and protonemata, the growth rates observed for pollen tubes are very fast, with lily (Lilium formosanum) pollen tubes reaching growth speeds of 500 nm/s in vitro (Cardenas et al., 2008). Although they grow slower, the genetic tractability of Arabidopsis and tobacco (Nicotiana tabacum) has led to the popularity of these systems (Krysan et al., 1999; Jube and Borthakur, 2007).

1.2.1 Physcomitrella patens Protonema
Due to the established techniques such as transient RNAi (Bezanilla et al., 2003; Bezanilla et al., 2005), temperature sensitive mutants (Vidali et al., 2009), stable transformations (Bezanilla et al., 2005; Vidali et al., 2009; Liu and Vidali, 2011),
rapid growth (Menand et al., 2007; Furt et al., 2013), and regenerative properties (Menand et al., 2007; Liu and Vidali, 2011), the moss *Physcomitrella patens*, has emerged as one of the best models to study polarized growth in plants. Following germination from the spore, *P. patens* is composed of long filamentous protonema tissue that is required for development (Cove, 2005; Vidali and Bezanilla, 2012). Each filament is one cell thick and composed of many cells attached in series. Each cell is 20 μm wide and approximately 200 μm in length. The cells at the end of these filaments exhibit tip growth and are classified as either chloronema or caulonema (Menand et al., 2007). Chloronema contain densely packed chloroplasts and grow at a rate of 2 – 5 μm/hr (Furt et al., 2012). Caulonema have fewer chloroplasts and grow more quickly at a rate of 25 – 40 μm/hr (Furt et al., 2012). Later in development, these filaments will eventually form the leafy gametophore. Because of their faster growth relative to chloronema, and their vesicle enriched growth zone, caulonema are the preferred cell type to study tip growth.

**1.2.2 Arabidopsis Root Hairs**

Root hairs elongate from the meristem, increasing the root surface area, and are thought to be important for water and nutrient uptake (Mendrinna and Persson, 2015). They are a single cell thick, 11 μm wide, and can grow up to 800 μm in length at speeds of 80 μm/hr (Rounds et al., 2011). As with protomenata and pollen tubes, this innate structure makes these systems amenable to various live cell microscopy techniques. Root hairs have emerged as an excellent model for plant growth because they are not essential for plant viability (Rounds and
Bezanilla, 2013). This permits the wide use of mutational analysis to study the genes necessary in tip growth (Schiefelbein and Somerville, 1990). Similar to pollen tubes and caulonema, roots hairs lack an outer cuticle layer and are therefore amenable to various pharmacological studies.

### 1.3 Organelle Distributions

Organelle distribution has been well established in tip-growing cells through electron micrographs, and their respective motilities have been determined through fluorescence studies (Lancelle and Hepler, 1992; McCauley and Hepler, 1992; Furt et al., 2012). In general, the apical dome where growth takes place is heavily enriched with Golgi, ER, and vesicles (Lancelle and Hepler, 1992; McCauley and Hepler, 1992; Bove et al., 2008; Furt et al., 2012). The concentration of vesicles at the apical dome highlights the requirement for the delivery of cell wall materials at the cell tip. Just behind the dome, the cell becomes more enriched in mitochondria and plastids. Further back from the cell apex, a large vacuole occupies much of the cell volume. In pollen tubes and root hairs, organelle motility is mostly conducted by cytoplasmic streaming (Mascarenhas and Lafountain, 1972; de Win et al., 1999; Verchot-Lubicz and Goldstein, 2010), a process in which flow of the cytosol may drive the motion of organelles. However, in *P. patens* there is no large organelle cytoplasmic streaming (Furt et al., 2012), indicating that this process is not essential for tip growth. Although not essential, it may be necessary to achieve enhanced growth rates (Tominaga et al., 2013).
1.4 Turgor Pressure and Wall Expansion

Mechanical models of cell growth and expansion have become important to cell biologists because they can make predictions about some of the controversial aspects of tip growth, including the spatial distribution of cell wall secretion. During growth, there is a constant internal turgor pressure that acts uniformly throughout the cell wall that provides the mechanical force necessary to extend the cell wall (Benkert et al., 1997). This internal pressure has been shown to reach up to 10 atmospheres and imposes stress on the cell wall (Baskin, 2005; Campàs and Mahadevan, 2009). The expansion of the cell wall is thus the result of this stress and the mechanical properties of the wall. Interestingly, the stresses predicted for tip-growing cells are opposite of the gradients of expansion seen during growth (Dumais et al., 2004). Specifically, stress analysis for thin pressurized shells suggests that the stresses on the cell wall would be maximal away from the cell tip near the cell shank (Figure 1.1D Eq. 1.1 and 1.2) (Campàs and Mahadevan, 2009). These stress, pressure, and curvature relationships were derived in (Campàs and Mahadevan, 2009) and take the following form,

\[ \kappa_s \sigma_{ss} + \kappa_\phi \sigma_{\phi\phi} = P \]  
\[ (Eq. 1.1) \]

\[ \kappa_\phi \sigma_{\phi\phi} = \frac{p}{2}. \]  
\[ (Eq. 1.2) \]
Here, $P$ is the internal turgor pressure on the shell, $\kappa_s$ is the curvature along the meridian, $\kappa_{\phi}$ is the curvature along the circumference, $\sigma_{ss}$ is the stress along the meridian, and $\sigma_{\phi\phi}$ is the stress along the circumference. Eqs. 1.1 and 1.2 indicate that the cell wall properties must exhibit maximum extensibilities at the cell tip to permit wall strain in regions of lower stress. Since the relationship between wall stress, curvature, and turgor pressure are independent of the material properties of the cell wall these Eq.1.1 and Eq. 1.2 vary minimally across most models.

Strain rates associated with wall expansion for pollen tubes and root hairs have been well characterized experimentally (Shaw et al., 2000; Dumais et al., 2004; Rojas et al., 2011), however, the exact rheology of the cell wall that relates stress and strain has been modeled in a variety of ways. Specifically, the wall has
been modeled as either an elastic (Fayant et al., 2010), viscous (Campàs and Mahadevan, 2009; Rojas et al., 2011), or viscoplastic (Dumais et al., 2006; Kroeger et al., 2011) material. When modeled elastically, there is no time dependence of deformation and the cell wall deforms instantly when a stress is applied (Fayant et al., 2010). Through cyclic loading and remeshing of an elastic finite element model, it was shown that the elastic modulus—how resistant a material is to stress-induced deformations—of the wall had to be reduced toward the cell tip to recapitulate the appropriate pollen tube morphology (Fayant et al., 2010). If this gradient of elastic modulus was too steep, the model predicted narrowing of the cell tip, while shallower gradients predicted ballooning of the cell tip (Fayant et al., 2010).

Viscous models of the cell wall assume that the rate of irreversible wall deformation is inversely proportional to the viscosity of the wall. This implies that the turgor pressure of the wall permanently deforms the cell wall at a particular rate. By monotonically decreasing the viscosity and monotonically increasing the secretion rate of the wall toward the cell tip, it is possible (even in the presence of nonmonotonic curvatures) to recapitulate a wide range of tip-growing morphologies (Campàs and Mahadevan, 2009). Furthermore, scaling laws derived by Campàs and Mahadevan (Campàs and Mahadevan, 2009), i.e.,

\[
\frac{R}{R_A} \sim \left(\frac{a}{R_A}\right)^{2/3}
\]  

(Eq. 1.3)

predict that the geometry of the cell is related to the effective size of the secretion zone. Here \( R \) is the radius of the cell, \( R_A \) is the radius of curvature at the cell tip, and \( a \) is the effective size of the secretion zone (Campàs and Mahadevan, 2009).
This implies that more pointed cells require secretion zones that get larger relative to their radius of curvature.

Viscoplastic models differ from viscous models because they allow irreversible deformations only above a critical yield stress (Dumais et al., 2006). Below this critical yield stress, they deform elastically (Dumais et al., 2006). With a viscoplastic anisotropic model of the cell wall material properties and a viscosity gradient that decreased toward the cell tip, Dumais et al. were able to produce a variety of relevant cell morphologies (Dumais et al., 2006). Unlike the previous viscous model (Campàs and Mahadevan, 2009), blunted cells with nonmonotonic curvatures could be produced with non-monotonic viscosity gradients.

Taken together these modeling efforts have begun to describe how secretion, turgor pressure, and the mechanical properties of the cell wall work in concert to achieve the morphologies of tip-growing cells. Importantly, these models describe the possible functional forms of wall secretion and wall viscosity. Additionally, these models can also make predictions of these functional forms in the case of mutant morphologies. These types of predictions are important because the spatial distribution of wall secretion is one of the more controversial topics in tip growth (Grebnev et al., 2017). There are two competing hypotheses; one is that secretion is maximal at the cell tip (Campàs and Mahadevan, 2009); another is that secretion is maximal in an annular region distal to the cell tip (Bove et al., 2008; Zonia and Munnik, 2008). With more experimental validation of the modeling assumptions in each of the previously described models, which to the
best of our knowledge has not been done, it will be possible to predict the true secretion profile more accurately.

1.5 Wall Composition

Polarized secretion is thought to be controlled by the delivery of Golgi derived vesicles containing cell wall materials and wall loosening enzymes to the growing apex of the cell wall. Although there remains no canonical marker for exocytosis, the area of exocytosis has been inferred by examining the composition of the cell wall (Fayant et al., 2010; Luo et al., 2017).

In pollen tubes, the cell wall is composed of three main polysaccharides, namely, pectin, cellulose, and callose (Fayant et al., 2010). Pectin stiffness is regulated by the demethylation of pectin via pectin methylesterases (Li et al., 1994). Upon demethylation of pectin, the negatively charged pectin attracts calcium ions which promote crosslinking of the pectins and makes them rigid (Bosch and Hepler, 2005; Rojas et al., 2011). The antibodies JIM5 and JIM6 selectively label methylated and de-methylated pectin respectively, and have been used to show that the apical dome of growing pollen tubes is highly methylated (Fayant et al., 2010). GFP fusions of pectin methylesterases have also revealed localization along the apical cell wall (McKenna et al., 2009). Moreover, fluorescence increases of these fusions and increases in cell wall thickness were shown to predict cell growth rates indicating that exocytosis of these enzymes can predict growth rates (McKenna et al., 2009). Pectin labeling with propidium iodide shows that actin is important for the focusing of pectin deposition at the cell tip (Rounds et al., 2014). In root hairs and moss protonemata, cellulose is more
prominent and may play a more important role in tip growth than pectin. In root hairs, it is thought to be loosened by expansins, which are localized to the tip of growing root hairs (Baluska et al., 2000). In moss protonemata, it was recently shown that cellulose synthase inhibitors result in disruption of caulonemata growth (Tran et al., 2018).

1.6 Exocytosis
To facilitate polarized growth, the plant cell is required to quickly deposit a significant amount of wall materials. It has been estimated that pollen tubes, protonemata, and root hairs need hundreds of vesicle fusion events per second to deposit the appropriate amount of wall materials during growth, Figure 1.2 (Bove et al., 2008; Ketelaar et al., 2008; Bibeau et al., 2017). Because exocytosis is such a fundamental process in tip growth, significant efforts have been made to visualize and measure this process.

One common approach is fluorescent labeling with FM dyes to show the localization of membranes (Bove et al., 2008). Using pulse-chase experiments with two different FM dyes in tobacco pollen tubes, it was shown that the pulsed dye could initially be found at the cell apex while the original dye also labeled the flanks of the cell (Zonia and Munnik, 2008). Based on this fluorescence localization it was determined that endocytosis occurs apically and that exocytosis occurs along the cell flanks (Zonia and Munnik, 2008). However, it is possible that exocytosis also occurs at the cell apex since both dyes remain apically localized throughout the time course.
Other markers include fluorescently labeled proteins involved in vesicle trafficking. These include t-SNARES, which are thought to be necessary for vesicle tethering during fusion of vesicles with the plasma membrane (Sanderfoot et al., 2001; Sanderfoot, 2007; El Kasmi et al., 2013). An alternative approach based on membrane proteins uses fluorescence recovery after photobleaching (FRAP) of GFP fused to receptor like-kinases (RLK) to follow protein movement; this analysis shows that recovery is fastest at the cell apex. Since RLKs are thought to be deposited on the plasma membrane via exocytosis, this suggests that the delivery of membrane proteins is most prominent at the cell tip (Lee et al., 2008). Another potential biomarker for endocytosis is the localization of ROP1

**Figure 1.2.** Vesicle fusion events are necessary to sustain polar growth in moss. Top (A) and side (B) views of scale model of the number of vesicle fusions (blue) necessary to sustain polarized growth. Vesicles are 80 nm in diameter. Of the 189 fusion events (see section 3.3.4), shown here in this cartoon, only 9 are required to sustain the amount of membranes needed for growth.
GTPase, which is thought to regulate the dynamics of apical actin and vesicle targeting (Gu et al., 2005; Lee et al., 2008).

Given the elucidation of various protein markers for exocytosis, another promising technology that could be used to identify endocytosis and exocytosis is the use of photo convertible fluorescent fusions (Luo et al., 2016). By photo converting a specified region of the plasma membrane protein it is possible to determine the rates at which this protein of interest is endocytosed and exocytosed (Luo et al., 2016). Through the use of this photo convertible technique, the distribution of fluorescent fusions, and exocytosis mutants, Luo et al. were able to build and constrain a mathematical model for chemically guided pollen tube growth (Luo et al., 2017). This model incorporates the feedback loops involved in ROP1 GTPase mediated exocytosis, pectin methylation, and cell wall mechanics, to recapitulate cell growth in response to extracellular signaling gradients (Luo et al., 2017). The model predicts that the wild type rate of exocytosis is required for the apical localization of ROP1 and that a 55% reduction of exocytosis results in broadening of ROP1 at the cell apex and wider slower growing cells. Although this model makes several assumptions, such as the nonlinear relationship between ROP1 concentration and the rate of exocytosis, it is the best available model for understanding the complex feedback between signaling, exocytosis, and tip growth in pollen tubes.

1.7 Endocytosis
To sustain cell expansion, the limiting factor is the delivery of wall materials not the delivery of membranes; thus an excess of membrane material is likely delivered to
the cell apex during exocytosis (Bove et al., 2008; Ketelaar et al., 2008). For this reason, it has been hypothesized that endocytosis plays an important role in the recycling of these membranes and other proteins (Grebnev et al., 2017). Although little research has been done on endocytosis in *P. patens* and root hairs, there has been significant, albeit controversial, research done in pollen tubes. This controversy stems from the debate regarding the spatial control of endocytosis. In one model it is believed that endocytosis takes place at the extreme apex of the pollen tube (Bove et al., 2008; Zonia and Munnik, 2008), while in another, it is thought that endocytosis happens behind the cell apex, along the flanks of the cell (Derksen et al., 1995; Zhao et al., 2010; Feng et al., 2016).

Similar to exocytosis, early studies on endocytosis used membrane labeling FM dyes. One study with FM4-64 in lily pollen tubes suggested that membranes are rapidly endocytosed along the cell flank within one minute and then recycled in 15 minutes into the secretory pathway at the cell apex (Parton et al., 2001). Although the initial internalization of the dye was not observed at the cell apex, it is difficult to interpret the specific location of this internalization. The localization of clathrin-mediated endocytosis at the cell flanks was determined by labeling clathrin heavy chains (Zhao et al., 2010) and clathrin light chains (Feng et al., 2016). In support of apical endocytosis, pulse-chase with preloaded FM1-43 and pulsed FM4-64 showed that the first colocalization of dyes appeared 1-2 minutes following the pulse at the cell apex (Zonia and Munnik, 2008). FRAP of pollen tubes preloaded with FM1-43 showed that the membranes at the cell apex recovered more slowly than the membranes at the cell flanks (Bove et al., 2008).
This fast turnover at the cell flanks was interpreted to be rapid exocytosis. However, this faster recovery could be confounded by the fact that the extreme apex is farther away from unphotobleached membranes than the cell flank. Based on this conflicting evidence more work is required to determine the exact location of endocytosis during polarized growth. Furthermore, the interpretation of FM-dye experiments would greatly benefit from modeling approaches. Models for receptor-mediated endocytosis (Birtwistle and Kholodenko, 2009; Layton et al., 2011) need to be adapted to better interpret how the FM-dye should label membranes once it is introduced into the cell.

1.8 The Cytoskeleton

1.8.1 Actin
The establishment of polarity is essential in tip growth; it has largely been hypothesized that the cytoskeleton plays a fundamental role. For this reason, filamentous actin has been extensively studied during tip growth. Work with the actin depolymerization agent latrunculin B—which functions by sequestering actin monomers—has shown that actin is required for tip growth (Vidali et al., 2001; Vidali et al., 2009; (Harries et al., 2005; Finka et al., 2007). Fluorescence localization of a yeast actin binding protein domain, Lifeact, has demonstrated that F-actin is focused to the growing cell tip of caulonemata, and also found along the cell cortex of the shank (Vidali et al., 2009). In root hairs, F-actin is bundled longitudinally away from the tip and less bundled focused filaments are found at the tip (Ketelaar et al., 2003). In pollen tubes, there is significantly less tip-focused actin, instead, a prominent actin fringe can be found microns behind the cell apex.
(Tiwari and Polito, 1988; Li et al., 2001; Raudaskoski et al., 2001; Vidali et al., 2001; Vidali et al., 2009). Modeling of the actin fringe orientation and vesicle dynamics demonstrated that this fringe (not apical F-actin) is sufficient to sustain the cytoplasmic streaming patterns in pollen (Kroeger et al., 2009). Total internal reflection microscopy demonstrated that the dynamics of FM dye-labeled vesicles was reduced by treatment with Latrunculin B (Wang et al., 2006). Latrunculin B treatment was also shown to abrogate the apical localization of fluorescently labeled pectin methyl esterase (PME) (Wang et al., 2013). Apical F-actin was also demonstrated to anticipate the direction of electric field-induced turning of pollen tubes (Daher and Geitmann, 2011). The differences in actin localization across model systems have confounded the understanding of the essential role of the actin cytoskeleton in tip growth. Thus understanding the mechanistic role of F-actin in tip growth remains an important question in the field.

1.8.2 Actin-Binding Proteins

The requirement for actin in polarized cell growth has also prompted the study of several actin-binding proteins. In *P. patens*, class II formins have been shown to be essential for tip growth; they localize to the cell apex, and rapidly polymerize actin filaments (Vidali et al., 2009). Overexpression of formins in root hairs (Deeks et al., 2005) and pollen tubes inhibits growth (Cheung and Wu, 2004), suggesting that the regulation of actin polymerization is necessary for tip growth. Work with the monomeric actin binding protein, profilin, has shown that profilin actin-binding is necessary to maintain polarized growth in *P. patens* (Vidali et al., 2007). This suggests that maintaining the pool of monomeric actin is important for polarized
growth. A range of other actin regulating proteins have been shown to be important for growth, these include, actin depolymerizing factor (ADF) (Augustine et al., 2008), actin-interacting protein 1 (AIP1) (Augustine et al., 2011), and the ARP2/3 complex (Harries et al., 2005). Taken together these studies suggest that F-actin must be tightly regulated to facilitate growth.

1.8.3 Myosin XI

The established importance of F-actin and its known regulators allowed for the natural hypothesis that myosin XI (Madison and Nebenfuhr, 2013), a myosin V homolog in plants, transports cell wall material along F-actin to the growing cell edge during tip growth. Although myosin V walks processively in a head over head manner in yeast, only the tobacco myosin XI is the only plant myosin XI shown to be processive (Tominaga et al., 2003). To better understand the function of myosin XI in plants, more mechanistic studies are needed.

Work with chimeric myosin XI showed that its motor domain could control the rate of cytoplasmic streaming and act as a plant size determinant in Arabidopsis (Tominaga et al., 2013). In P. patens, fluorescently labeled myosin XI was shown to localize to the cell apex and be essential for tip growth (Vidali et al., 2010). Work with early-stage filament branches suggested that myosin XI is important for actin organization and not actin dynamics (Vidali et al., 2010). However, these claims can be better supported with conditional loss of function myosin XIs and fully formed filamentous cells. Cross-correlation analysis of myosin XI and F-actin apical fluorescence fluctuations demonstrate that myosin XI fluctuations can predict increases in F-actin fluctuations (Furt et al., 2012),
indicating that myosin XI can dynamically impact F-actin. Until an interaction between full-length myosin XI and F-actin can be seen in vivo, the feedback between these two components of the cytoskeleton remains speculative.

In Arabidopsis, there are 13 myosin XI isoforms that complicate reverse genetic studies (Reddy and Day, 2001). Nevertheless, knockout lines (Ojangu et al., 2007) and dominant negative approaches (Peremyslov et al., 2008) have demonstrated that myosin XI is important for tip growth in root hairs.

The cargo for myosin XI has been the subject of several studies in plants. In P. patens, myosin XI was shown to co-localize to the growing cell tip and fluctuate in phase with vesicles fluorescently labeled with the v-SNARE VAMP72 (Furt et al., 2013). In root hairs, myosin XI-K was found to localize with and influence the dynamics of secretory vesicle-associated membrane protein (SCAMP) labeled vesicles (Peremyslov et al., 2012; Park and Nebenfuhr, 2013). In both instances, this localization was only found at the cell tip, and no individual molecule binding was observed. Thus, to the best of our knowledge, no single molecule interactions have been shown between myosin XI and its cargo during polarized growth.

1.8.4 Microtubules
Similar to actin, microtubules have also been implicated in the regulation of polarized growth. As early as the 1980s, it was shown that microtubule-depolymerizing drugs lead to aberrant tip growth. Specifically, protonemata became swollen and in some cases produced more than one growing tip (Doonan et al., 1988). When treated with similar microtubule depolymerizing drugs, root
hairs exhibited a wavy growth pattern and also produced multiple tips (Bibikova et al., 1999). Fluorescent labeling of the microtubule subunit, α-tubulin, and the plus end binding protein, EB1, demonstrated that microtubules are oriented longitudinally and focus their plus ends toward the cell tip (Hiwatashi et al., 2014). This plus end focusing was shown to oscillate on time scales similar to the observed actin oscillations (Hiwatashi et al., 2014) and may suggest cross-talk between the two cytoskeletal systems.

1.9 The Potential Feedback Between Vesicles and the Cytoskeletal Systems

It has been shown that actin, its binding proteins, myosin XI, microtubules, and vesicles are all focused at the cell tip and are essential for growth. Furthermore, fluorescence fluctuations and phase relationships between actin, myosin XI, and vesicles have demonstrated that myosin XI fluctuations can anticipate actin fluctuations. Taken together this suggests that there is a complex feedback mechanism between myosin XI cargo delivery and actin regulation. One hypothesis that includes this feedback mechanism is the vesicle-organizing center model; which was first established in mouse oocytes (Schuh, 2011; Holubcova et al., 2013) and adapted later to tip growth (Furt et al., 2013). In this model, vesicles contain actin nucleators and can promote actin filament assembly. When myosin walks along actin filaments, the nucleators present on the vesicle promote the formation of more actin filaments, which promote more myosin binding causing a positive feedback loop in which a spike in myosin concentration at the tip can precede an increase in actin concentration (Figure 1.3). Once this reaches a
critical concentration, an additional factor such as an actin-severing protein might intervene to break the feedback loop and further control vesicle delivery.

**Figure 1.3.** Positive feedback of F-actin via the vesicle organizing center model
A) Vesicles (blue) anchored to an actin filament (orange) via myosin XI (red), nucleate actin. B) As the actin polymerizes more myosin XI carrying vesicles attach to actin. C) Positive feedback continues as more myosin XIs attach to actin filaments and more vesicles begin to nucleate the polymerization of more filaments.
1.10 Ca$^{2+}$ Signaling

Calcium is one of the key signaling molecules that has been shown to be important in the regulation of many of the aspects of polarized growth including actin-binding proteins, vesicle dynamics, and cell wall extensibility. In pollen tubes, cytoplasmic calcium exhibits a tip-focused gradient with a maximum concentration between $2 \rightarrow 10 \mu M$ that has been shown to oscillate with a period between 15 and 60 seconds (HoldawayClarke et al., 1997). These gradients have been shown to be essential for pollen tube growth and can even control the direction of growth. The formation of these gradients has been long thought to be a product of membrane-bound calcium channels. Patch clamp analysis revealed that pressure on the cell membrane of pollen tubes could induce calcium channel activity (Dutta and Robinson, 2004). This indicates that stretch-activated calcium channels may be important for the regulation of cytosolic calcium concentrations. Although a few genes have been identified as potential stretch-activated channels (Nakagawa et al., 2007), no definitive candidates have been elucidated (Steinhorst and Kudla, 2013).

Calcium gradients are thought to regulate the activity of the uniformly distributed actin-binding proteins, which include profilin, L1LIM1, and villins (Cheung and Wu, 2008; Wang et al., 2008; Zhang et al., 2010). By modulating the activity of these proteins, calcium can indirectly influence actin dynamics. Calcium has also been hypothesized to control vesicle trafficking by blocking myosin activity via its calmodulin domain (Cai and Cresti, 2009) or by promoting exocytosis (Battey et al., 1999). In the cell wall, calcium is involved in the maintenance of cell wall rigidity by crosslinking de-esterified pectins (Bosch and Hepler, 2005). The
spatial regulation of this crosslinking allows the tip of the cell to be more extensible than the shank.

1.11 Modeling Cell Polarization
The feedback between endocytosis, exocytosis, calcium signaling, and the cytoskeleton to maintain an intrinsic polarity has been the subject of various modeling efforts (Bartnickigarcia et al., 1989). One of the earliest models for polarized cell growth is the vesicle supply center, VSC, model for fungal hyphae (Figure 1.4A) (Bartnickigarcia et al., 1989). In this model, it is assumed that the number of vesicles, $N$, emanate along ballistic trajectories from a vesicle rich zone behind the tip of a growing cell. These vesicles then fuse with the cell wall and contribute to the elongation of the cell. As the cell grows, this vesicle rich zone translates at a velocity, $V$, and maintains a constant distance from the cell edge. Assuming that the vesicle supply center is at the origin and that growth happens along the $y$-axis, the morphology of fungal hyphae can be described as,

$$y = x \cot \frac{xV}{N}. \quad (Eq. 1.4)$$

Here $y$ describes the morphology of the cell wall as a function of its position along the $x$-axis.

Breaks in the initial cell growth symmetry can be explained by the position of the vesicle supply center. When positioned close to one edge of a spherical cell, this edge closest to the VSC captures the bulk of the vesicles produced by the VSC. This allows for the preferential deposition of wall materials in that zone and leads to polarized expansion. Since the purely ballistic motion of vesicles may be unrealistic, the diffusive motion of vesicles from the VSC were still able to predict
polarized fungal growth (Tindemans et al., 2006). However, the predicted morphologies for this model were more broadened than the typical hyphae growth cone (Tindemans et al., 2006).

Although the VSC model can well explain fungal growth, it imposes the position of the vesicle supply center. To accurately model cell polarization the position of the vesicle supply center would have to be an emergent property of the model. This polarization has been previously modeled by either the active transport of signaling molecules to the plasma membrane or by reaction-diffusion systems on the cell membrane. Modeling of the polarization regulator, Cdc42, in budding yeast has given valuable insight into the feedback between endocytosis, exocytosis, and the cytoskeleton (Marco et al., 2007). Here the concentration of Cdc42 on the membrane can be modeled as,

\[
\frac{df}{dt} = D_f \nabla^2 f - \left[ e_a \chi + \frac{e_a}{\alpha} \tilde{\chi} \right] f + h F_{cyto} \frac{\chi}{\tilde{\chi}}. \tag{Eq. 1.5}
\]

Where \( f \) represents the concentration of Cdc42 on the membrane, \( \nabla^2 \) is the Laplace operator for diffusion along the membrane, \( \chi \) is the area on the membrane where transport occurs, \( \tilde{\chi} = 1 - \chi \), \( e_a \) is the rate of endocytosis in the region of transport, \( \alpha \) is the ratio of the \( e_a \) to the rate of exocytosis elsewhere on the membrane, \( h \) is the rate of directed transport, and \( F_{cyto} \) is the concentration of cytosolic Cdc42 (Figure 1.4B). To maintain a sharp localization of Cdc42, the model predicts that a critical rate of endocytosis must be achieved. When endocytosis happens too quickly, Cdc42 is forced into the cytosolic pool; conversely, when endocytosis is too slow, Cdc42 becomes homogenously dispersed throughout the membrane.
Further work that modeled Cdc42 polarization with the inclusion of vesicles containing Cdc42, demonstrated that actin delivery of vesicles could not maintain Cdc42 polarization (Layton et al., 2011). With the steady-state assumption that endocytic rates had to equal exocytic rates, it was shown that apically focused exocytosis only diluted apical membrane concentrations. To maintain apical Cdc42 localization, a concentrating mechanism had to be imposed on all endocytic vesicles. For this reason, there remains a need for more modeling on directed transport and the maintenance of membrane polarity.

Figure 1.4. Models for polarization. A) Vesicle Supply Center (VSC) model for fungal hyphae growth. Vesicles emanate from a VSC (gray circle) and fuse to the cell wall (gray outline). The VSC maintains its distance from the cell tip and its position influences cell morphology. B) Cytoskeleton directed exocytosis cell polarization model of the membrane protein Cdc42. Orange arrows indicate F-actin mediated exocytosis, straight green arrows indicate endocytosis, curved green arrows indicate recycling, green circles with red outline indicate Cdc42 labeled vesicles, and the red outline indicates the concentration of Cdc42 on the membrane.

Neglecting the requirement for active transport, reaction-diffusion systems have been shown to maintain polarity. It was shown that cells with an elliptical shape could induce transient polarity with a simple membrane reaction (Rangamani et al., 2013),

\[ A + X \rightleftharpoons B \]  \hspace{1cm} (Eq. 1.6)
Here, $A$ is a cytoplasmic protein and can bind to the membrane component $X$ to form the new membrane component $B$. These transient events may be important in various signaling cascades and underscore the importance of cell shape in \textit{in vivo} reactions. More complex reaction-diffusion systems have also been shown to achieve equilibrium membrane polarization (Semplice et al., 2012) but these complex models are challenging to constrain given the current understanding of tip growth in plants.

1.12 Oscillations

It been shown extensively in pollen tubes that growth rates (Holdaway-Clarke and Hepler, 2003; Cardenas et al., 2008), F-actin concentrations (Hwang et al., 2005), wall thickness (McKenna et al., 2009), calcium concentrations (Pierson et al., 1996), and exocytosis rates (McKenna et al., 2009), all oscillate during growth. The phase relationships of these oscillations were outlined in (Hepler et al., 2013). Unfortunately, a direct mechanistic link between each of these signals has remained evasive because studies that try and block any one of these signals lead to the suppression of all the other oscillations.

This suggests that these signals may all be dependent on each other through feedback mechanisms. Interestingly, positive and negative feedback loops, along with nonlinearities and time delays, are among the minimal requirement for producing sustained oscillatory behavior within a system (Novak and Tyson, 2008). Modeling efforts in pollen tubes have demonstrated that oscillatory behavior can be recapitulated under various conditions without the requirement for an oscillatory driver such as oscillating turgor pressure (Kroeger et al., 2008; Irvine et al., 2009;
Liu et al., 2010; Kroeger et al., 2011; Rojas et al., 2011). Through the coupling of a viscoelastic model of wall expansion, stress-induced calcium channels, and calcium-mediated vesicle deposition it was shown that wall thickness, growth rates, and calcium concentrations could all oscillate at steady state (Kroeger et al., 2011). Liu et al. showed that a two-compartment model of ion dynamics and cell growth could produce ion oscillations at the cell tip (Liu et al., 2010). Yan et al. demonstrated that the feedback mechanisms between F-actin, the small GTPase ROP1, and calcium, along with the appropriate time delay, could produce oscillations in the concentrations of calcium and active ROP1 (Irvine et al., 2009). Modeling of the feedback mechanism between pectin deposition, chemical crosslinking, and mechanical deformation was shown to be sufficient to reproduce to the experimental phase relationships between growth rates, wall morphology, wall thickness, and calcium concentrations (Rojas et al., 2011). Taken together these models highlight potential mechanisms that could induce emergent oscillations during tip growth.

In moss, growth rates are too slow to show oscillations, but non-periodic cytoskeletal oscillations at the cell tip have been observed (Furt et al., 2013). More characterization of this aperiodicity is required to better understand why these cytoskeletal oscillations appear to be uncoupled from growth rates. However, aperiodic oscillations may be the result of a chaotic system, which can easily emerge from systems with coupled oscillators (Novak and Tyson, 2008).
1.13 Single Particle Tracking

Mathematical modeling has contributed several important insights into the field of polarized cell growth in plants. However, the bulk of these models coarse-grain the actin cytoskeleton with imposed secretion profiles or convective terms. Thus there remains a need to incorporate the true dynamics and kinetics of the cytoskeleton in existing models. Specifically, this would involve modeling the dynamic interactions between actin motors, their cargo, and filamentous actin. In order to build such a model, significant work must be done to parameterize these complex interactions. For the remainder of this chapter we will discuss some of the existing in vivo techniques that will help measure these parameters.

Since the dynamic coordination of the cytoskeleton and its constituents have been implicated in polarized growth, studying live cell protein and vesicle dynamics has become increasingly important. Several fluorescent assays have emerged as important tools to probe relevant physical constants such as diffusion coefficients, protein concentrations, and chemical rate constants. Measuring these constants becomes necessary to constrain predictive physical modeling and can better establish the feasibility of potential hypotheses.

One of the most common ways to measure protein dynamics is single particle tracking; in which individual fluorescent proteins can be resolved under the microscope and tracked with time-lapse imaging. With particle tracking, it has been possible to show that individual molecules interact in vivo. Particle tracking has also helped uncover the dynamic nature of the proteins involved in tip growth (van Gisbergen et al., 2012). Although particle tracking has helped shape the way we view tip growth, it is not without limitations. When protein densities are too...
high, single particle tracking is not possible (Chenouard et al., 2014). Additionally, following image acquisition, algorithmic approaches are required to track particles within each image. This involves particle identification, which is often done with Gaussian fitting, image filtering, or other methods (Chenouard et al., 2014). Once the particles are recognized on each image, a linking algorithm is needed to determine the most likely path taken by each particle (Jaqaman et al., 2008). This is non-trivial and can be confounded by particle merging, splitting, and moving out of focus (Jaqaman et al., 2008). Furthermore, increasing particle densities compounds these issues.

To illuminate fewer particles in the field of view, Variable Angle Epi-fluorescence Microscopy (VAEM) is often used (Konopka and Bednarek, 2008). By hitting a coverslip with a laser at an oblique angle, an evanescent field can be used to illuminate a thin portion of the cell. Unfortunately, this cannot be used at the cell tip during polarized growth because the tip curves away from the coverslip and away from the evanescent field. For this reason, additional assays are necessary to probe protein dynamics at the cell tip during polarized growth.

1.13.1 Mean Squared Displacement
When particle tracking is permitted, Mean Squared Displacement is the one of the primary tools used to measure protein diffusion. MSD describes how far a diffusing particle can be expected to deviate from its origin at a given time. In one dimension this can be written as (Phillips, 2013),

\[ \langle x^2 \rangle = 2D_{eff}t. \]  \( (Eq. 2.1) \)
Here $D$ is the diffusion coefficient, and $\langle x^2 \rangle$ represents the average squared distance a particle is expected to travel from its origin in the time $t$. In special cases termed anomalous diffusion, mean squared displacement does not exhibit a linear dependence on time. Instead, it can be represented as a power law,

$$\langle x^2 \rangle \propto D t^\alpha$$

(Eq. 2.2)

where the exponent $\alpha$ describes the nature of the nonlinearity (Metzler et al., 2014; Kepten et al., 2015). For $\alpha < 1$, the particle’s motion is sub-diffusive, indicating that the particle may experience confinement at long times (Metzler et al., 2014). For $\alpha > 1$, the particle’s motion is super-diffusive and indicates that the particle may experience ballistic motion, possibly via active transport (Metzler et al., 2014).

By averaging the displacements of many particle trajectories, it is possible to measure effective diffusion coefficients and characterize protein dynamics with $\alpha$ by fitting to experimental data. However, many particle trajectories are needed for sufficient averaging of large displacements. Additionally, the finite size of the cell should produce sub-diffusive exponents at length scales on the order of the size of the cell. For both of these reasons, long displacements should be ignored during the analysis of MSD. Unfortunately, determining the specific length scales to ignore can be difficult and in some cases arbitrary. Another limitation in MSD analysis is that it cannot predict the state of a given protein at a specific time because it is an ensemble approach. For these reasons, there is a growing need for additional complimentary methods for analyzing particle-tracking data.
1.13.2 Hidden Markov Models
One potential model that has shown promise in analyzing particle trajectories is the Hidden Markov Model (HMM) (Rabiner, 1989; Roding et al., 2014). From the field of artificial intelligence, HMMs are used to probabilistically determine the hidden state of some system given some observable information from that system. Roding et al., showed that HMMs could be used to predict if myosin is moving processively along an actin filament or moving randomly via Brownian motion (Roding et al., 2014). HMMs also show promise in that they predict the probability of transitioning between hidden states. In Roding et al. HMMs were used to determine the probability of a myosin coming onto or off an actin filament. Given these transition probabilities, it is possible to infer information about the chemical on and off rates of a protein of interest. Similar to MSD, HMMs require many protein trajectories for accurate analysis. Furthermore, the accuracy of HMMs and MSD is heavily reliant on the fidelity of the protein tracking data. In instances where reliable protein tracking is not possible, additional techniques are required to measure protein dynamics.

1.14 Fluorescence Correlation Spectroscopy
One of the most widely used techniques to assess protein dynamics is fluorescence correlation spectroscopy (FCS) (Elson, 2011; Fitzpatrick and Lillemeier, 2011). Unlike particle tracking, FCS can be conducted when individual particles cannot be resolved. In this technique, a resonant scanner is typically used to acquire fluorescence fluctuations on nano and micro second time scales. Since acquisition happens on time scales faster than particle motion, the fluorescence fluctuations remain correlated for some time. The length of this time is directly
related to the rate of the particle motion and is determined by applying the autocorrelation function to the intensity fluctuations (Elson, 2011; Fitzpatrick and Lillemeier, 2011).

\[
G(\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle I(t)^2 \rangle} \quad (Eq. 2.3)
\]

Here, \( I(t) \) is the time-dependent intensity fluctuations within the confocal volume, \( \tau \) is a time shift, and \( \langle \rangle \) denotes the time average. Several analytical models have been made to link this autocorrelation function to a wide array of particle motions in one, two and three dimensions (Fitzpatrick and Lillemeier, 2011). One of the of these models links fluorescence fluctuations to unconjugated Brownian motion in three dimensions (Fitzpatrick and Lillemeier, 2011), i.e,

\[
G(\tau) = 1 + \frac{1}{N_p} \frac{1}{(1+\tau/\zeta)^2} \frac{1}{\sqrt{(1+\tau/\zeta)^2}} \quad (Eq. 2.4)
\]

Here \( N_p \) is the number of particles in the confocal volume, \( \zeta = w^2/4D \), \( w \) is the \( e^{-2} \) confocal beam radius along the x and y-axes, \( \kappa = w/w_z \), \( w_z \) is the \( e^{-2} \) beam radius along the z-axis, and \( D \) is the diffusion coefficient of the molecule of interest. More complicated models have been shown to characterize binding kinetics.

### 1.15 Raster Image Correlation Spectroscopy

Similar to FCS in that it utilizes correlation methods to extract the dynamic information of proteins, is raster image correlation spectroscopy (RICS) (Digman et al., 2005; Brown et al., 2008; Rossow et al., 2010; Clark et al., 2016). Unlike FCS, RICS does not require a resonant scanner and can be easily conducted on most scanning confocal microscopes. RICS utilizes the hidden temporal
information within a confocal image by determining the spatial autocorrelation function for each image (Digman et al., 2005).

\[
G(\xi, \psi) = \frac{\langle \delta i(x,y) \delta i(x+\xi, y+\psi) \rangle_{x,y}}{\langle i(x,y) \rangle_{x,y} \langle i(x,y) \rangle_{x,y}} \quad (Eq. 2.5)
\]

Where \(i(x,y)\) is the intensity at each pixel in the image, \(\xi\) is the shift in x, \(\psi\) is the shift in y, and \(\delta i = i - \langle i \rangle\). For large stacks of images, \(G(\xi, \psi)\) can be averaged across images.

During image acquisition, a scanning confocal microscope rasters a laser across a region of interest. Since rotating the galvanic mirrors inside the confocal does not happen instantaneously, there is a time delay between the acquisitions of each pixel. This time delay allows for spatial correlation between pixels as particles move during image acquisition. Analytical models have been developed to relate the spatial autocorrelation function and Brownian motion. In three dimensions the relationship can be characterized by the following (Digman et al., 2005),

\[
G_{RICS} = G(\xi, \psi)S(\xi, \psi) + b \quad (Eq. 2.6)
\]

\[
G(\xi, \psi) = \frac{\lambda}{N} \left( 1 + \frac{4D(\tau_p \xi + \tau_i \psi)}{w_0^2} \right)^{-1} \left( 1 + \frac{4D(\tau_p \xi + \tau_i \psi)}{w_0^2} \right)^{-1/2} \quad (Eq. 2.7)
\]

\[
S(\xi, \psi) = \exp \left( -\frac{1}{2} \left[ \frac{(2D_\xi \xi)^2}{w_0^2} + \frac{(2D_\psi \psi)^2}{w_0^2} \right] \right). \quad (Eq. 2.8)
\]

\(G_{RICS}\) can be broken into a component related to the dynamics of the molecule of interest \(G(\xi, \psi)\), and a component related to the scanning of the confocal \(S(\xi, \psi)\). Here \(D\) is the diffusion coefficient, \(N\) is the number of particles in the imaging
volume, $\lambda$ is a correction factor for the illumination volume, $b$ is background, $w_o$ is the $e^{-2}$ waist of a three-dimensional Gaussian function in $xy$, $w_z$ is the $e^{-2}$ beam radius in $z$, $\delta_r$ is the pixel size, $\tau_l$ is the time between each line scan, and $\tau_p$ is the pixel dwell time.

RICS can also be extended to two-color imaging to infer the in vivo binding of molecules of interest (Digman et al., 2009; Sasaki et al., 2015). Termed cross-correlation RICS (ccRICS), this technique examines the amplitude of the cross-correlation between the two channels to detect binding. ccRICS is limited in that it offers a binary measurement for binding but cannot predict binding constants.

1.16 Space-Time Image-correlation and Cross-correlation Spectroscopy
Another type of fluorescence fluctuation analysis is Space-Time Image Correlation and Cross-correlation Spectroscopy (STICS) patio (Hebert et al., 2005). STICS utilizes spatial-temporal autocorrelations to characterize proteins that undergo slow directed transport and diffusion. This analysis has been used to identify flow fields of FM dye-labeled membranes in pollen tubes and demonstrated that the direction of these flow fields occurs in a reverse fountain shape (Bove et al., 2008). This methodology requires rapid image acquisition so that temporal correlations from image to image can be detected.

1.17 Number and Brightness Analysis
When image-to-image fluctuations are no longer correlated, another useful technique called Number and Brightness (N&B) can be implemented (Digman et al., 2008). This method uses confocal fluorescence fluctuations and a photon
counting detector to determine the concentration of proteins in solution along with their apparent brightness. This analysis is founded on the assumption that molecule number fluctuations in a given volume are Poisson distributed—which holds when proteins are freely diffusing. Because a Poisson distribution has an equal mean and variance, the brightness and concentration of a protein can be solved algebraically even in the presence of detector shot noise. Specifically, the number of molecules in the confocal volume \( n \), and the brightness of each molecule \( \varepsilon \) can be written as,

\[
n = \frac{\langle k \rangle^2}{\sigma^2 - \langle k \rangle} \quad (Eq. 2.9)
\]

\[
\varepsilon = \frac{\sigma^2 - \langle k \rangle}{\langle k \rangle} \quad (Eq. 2.10)
\]

Here \( \langle k \rangle \) is the average value at a specific pixel location for a stack of images and \( \sigma^2 \) is the variance. This technique can also be extended to non-photon counting detectors by measuring a conversion factor \( S \) that relates one photon detected to the number of digital levels produced by the electronics (Dalal et al., 2008).

By examining the cross brightness and cross variance between pixel intensities, it is possible to measure the \textit{in vivo} stoichiometry and localization of slow moving protein complexes (Digman et al., 2009). However, because this method relies on differences in the pixel distribution, it cannot be used in cases where there is uniform homogenous binding between two fast-moving species.

### 1.18 Fluorescence Recovery After Photobleaching

Another technique that has been used to study protein dynamics during polarized growth is Fluorescence Recovery After Photobleaching (FRAP) (McNally, 2008;
Loren et al., 2015). This technique takes advantage of the fact that fluorophores lose their fluorescence when they are exposed to a high-intensity laser pulse. With a scanning confocal microscope, it is possible to photobleach fluorophores within a user-specified region of interest. Then, after this bleaching event, unbleached fluorophores will move into the bleached region. This movement causes a transient fluorescence recovery. The speed and directionality of this recovery are then used to infer information regarding the dynamics of the protein of interest through model fitting. This technique is advantageous because it does not require a resonant scanner and works best when protein concentrations are high. For these reasons, FRAP has been used to study dynamics during polarized growth (Bove et al., 2008; Lee et al., 2008; Bibeau et al., 2017; Kingsley et al., 2017).

FRAP of diffusing species is often modeled by solving the diffusion equation in various dimensions with specific initial and boundary conditions. The most commonly used models are the analytical expression for FRAP in two dimensions with infinite cell boundary assumptions and circular bleach profiles (Axelrod et al., 1976; Soumpasis, 1983). However, due to the confined cell geometry at the cell tip imposed by the cell wall, it is unclear how applicable these models are in tip growing systems. Alternatively, a one-dimensional model with reflective boundaries at the cell tip could be used in cases where the bulk of fluorescence recovery happens in one direction (Phillips, 2013). Unfortunately, the size of tip-growing cells and the limited bleach volume are not likely to permit these assumptions in most microscope systems. Although there are significant modeling efforts that have incorporated several important aspects in FRAP including,
reversible photobleaching (Sinnecker et al., 2005; Mueller et al., 2012; Morisaki and McNally, 2014), finite confocal scan rates during photobleaching (Braeckmans et al., 2003; Deschout et al., 2010; Kang et al., 2010; Smisdom et al., 2011), confocal imaging (Smisdom et al., 2011), arbitrary bleach profiles (Blumenthal et al., 2015), and the appropriate cell boundaries (Loew and Schaff, 2001; Sbalzarini et al., 2005; Sullivan and Brown, 2011; Mai et al., 2013), it remains unclear how all of these effects can together influence fluorescence recovery. Because incorporating and understanding all of these effects would be time-consuming, as we will show quantitatively, we suggest that users trying to measure diffusion coefficient during tip growth use either the Virtual FRAP tool developed within the Virtual Cell software (Loew and Schaff, 2001) or the Digital Confocal Microscopy Suite (DCMS) software. The Virtual FRAP tool reads image stacks and incorporates the two-dimensional geometry of the cell and works best with low numerical aperture lenses. DCMS read image stacks and produces fluorescence recoveries in any arbitrary geometry with an point-spread-function but requires manual recursion to measure diffusion coefficients.

Fluorescence recovery after photobleaching has also been shown to be able to measure the rates of reactions in vivo for idealized cases. In most cases there is a simple reaction in which some unbound protein $U$ binds to free binding sites $S$ reversibly to form a bound complex $B$, $U + S \rightleftharpoons B$. Here it is assumed that the unbound protein $U$ is fluorescently labeled and retains its fluorescence when it is part of the complex $B$. This can be expressed as a system of reaction diffusion equations (Sprague et al., 2004).
Here \( k_{on} \) is the on rate, \( k_{off} \) is the off rate, \( \nabla \) is the gradient operator, and \( D \) is the diffusion coefficient of a specific protein denoted by the subscript. Sprague et al. devised and characterized analytical models for this reaction under the assumptions that the concentration of sites \( S \) is in equilibrium and immobile and that the bound complex \( B \) is also immobile (Sprague et al., 2004; Sprague and McNally, 2005). Later models were developed in which all of the components of the reaction were allowed to be mobile (Kang and Kenworthy, 2008; Kang et al., 2010). Although these models show great promise in measuring reaction rates, they make many of the same assumptions as typical diffusive FRAP models, such as infinite cell boundaries. Furthermore, the ability to measure the reaction rates with FRAP works only when the difference between \( D_B \) and \( D_U \) is significant.

1.19 Summary
Substantial modeling and experimental efforts have propelled the field of tip growth in plants. However, the role of the cytoskeleton has often been coarse-grained by simple secretion fluxes in most of these models. Although modeling the cytoskeleton as a directed flux is a good first approach, it provides little to no insight into the true nature and function of the cytoskeleton. To begin to quantitatively model the feedback between the cytoskeleton, secretion, the cell wall, and cell growth, significant efforts must be made to constrain several key cytoskeletal
parameters in this process. On the most basic level, the coordinated interactions
tween myosin XIa, secretory vesicles, actin nucleators, and the actin
cytoskeleton must be elucidated. These minimal requirements are outlined in
Figure 1.5. Measuring parameters like on and off rates of the cytoskeletal
components, protein diffusion coefficients, motor run lengths, and actin
polymerization rates will make it possible to model the role of the actin cytoskeleton
in plant polarized secretion for the first time. Because they lack large organelle
cytoplasmic streaming, moss protonemata are an excellent model system in which
these parameters can be measured.

Recent developments in the field of fluorescence dynamics will make
measuring these parameters possible. For example, approaches such as FRAP,
FCS, and RICS can be used to measure protein diffusion coefficients. However,
the accuracy of these techniques is heavily dependent on model selection, and the
assumptions inherent in each model. For example, infinite boundary models
should be avoided when measuring dynamics at the cell tip; instead more flexible
algorithmic models that take the cell shape into account should be used, as we will
show in section 2. Similarly techniques such as FRAP and FCS can be used to
measure binding kinetics. It is important to note that accuracy of these techniques
is improved when the theoretical diffusion coefficients of the candidate proteins are
substantially different. When the diffusion coefficients of the candidate proteins are
similar, variable angle epiflourescence microscopy coupled with particle tracking
can serve as a viable alternative. By tracking protein movement directly, observing
binding association times, and looking at the fraction of bound proteins, it is possible to quantitatively measure reaction kinetics in live cells.

Furthermore, protein tracking can also be used to determine how motor proteins like myosin XI interface with the cytoskeleton. Although ensemble averaging trajectories and plotting the MSDs can suggest the presence of super-diffusion, it can be difficult or impossible to directly infer more specific parameters with these techniques. To directly measure the interactions between the cytoskeleton and motor proteins, two-color fluorescence labeling of the cytoskeleton and motor proteins is possible. However, these techniques require accurate tracking of cytoskeletal filaments in vivo. Alternatively, hidden Markov models can be used to statistically infer run lengths, off-rates, and on-rates of motor proteins on actin filaments.

Once these necessary parameters are measured, the cytoskeleton can be incorporated into existing growth models that take into account the turgor pressure induced deformation of the cell wall. Specifically, it will be possible to determine the role of actin in maintaining the polarity of the cell and its role in vesicle secretion. It will also be possible to understand how and why F-actin and myosin XI fluctuations—seen at the cell tip—emerge in this system. More generally these models will begin to provide the much-needed mechanistic information regarding the cytoskeleton’s role in polarized growth. To allow for accurate measurements of diffusion coefficients at the cell tip, we will construct a model for fluorescence recoveries in tip growing cells, section 2.
Figure 1.5. Modeling parameters important for cell growth. Cartoon model of polarized cell growth where vesicles with an actin nucleator are carried along existing actin filaments to the secretion zone at the cell tip. At the cell tip vesicles fuse with the cell membrane at a given rate. Fusion provides more wall materials at the growing cell edge and locally adjusts the Young’s modulus of the cell wall. Turgor pressure drives expansion only at the cell apex. To better understand how this process is coordinated in silico various parameters must be measured. These parameters are depicted above and include, the myosin Xi vesicle kinetics, the myosin XI actin kinetics, actin nucleator vesicle kinetics, actin polymerization and depolymerization rates, vesicle fusion kinetics, the Young's modulus of the cell wall, the cell growth rate, protein diffusion coefficients, the secretion zone size, and the intracellular turgor pressure.
2 Understanding Boundary Effects and Confocal Optics Enables Quantitative FRAP Analysis in Tip Growing Cells

2.1 Abstract
Fluorescence Recovery After Photobleaching (FRAP) is a valuable tool used by cell biologists to study the diffusion and binding kinetics of vesicles, proteins, and other molecules in the cytoplasm, nucleus or cell membrane. Specifically, FRAP has the potential to elucidate protein dynamics during tip growth. With an understanding of the spatial coordination of the cytoskeleton and various membrane compartments, it is possible to build more mechanistic models of tip growth. While many FRAP models have been developed over the past decades, it remains unclear how the confined geometry of tip-growing cells influence diffusion measurements. To this aim, we first used analytical modeling of FRAP to demonstrate that the cell apex can influence fluorescence recovery measurements. To account for cell shape effects, we developed a three-dimensional computational model of the FRAP process that incorporates particle diffusion, cell boundary effects, and the optical properties of the scanning confocal microscope. We then used this model to measure the diffusion coefficient of unconjugated 3mEGFP in the tip growing cells of Physcomitrella patens. As we will show towards the end of the chapter, with an accurate method of measuring diffusion at the cell tip, it will be possible to determine the role of diffusion in polarized growth.
2.2 Introduction

Due to the popularity of confocal laser scanning microscopes, FRAP has emerged as a prominent technique to measure protein mobility, and throughout the past decade has appeared in over 150 publications annually (Loren et al., 2015). During a typical FRAP experiment; a cell expressing a fluorophore of interest is subjected to a high-intensity laser pulse, which permanently abolishes the fluorescence properties of the fluorophore—a process called photobleaching. This laser pulse is specifically localized to a predetermined Region Of Interest (ROI) inside the cell, and over time fluorophores not subjected to the bleach move into the ROI, leading to a recovery of the local fluorescence. The rate and directionality of this fluorescence recovery can then be used—via a model—to determine an array of important characteristics of the molecule of interest such as diffusion coefficients, binding kinetics, and the number of dynamic states of the fluorophore. In the context of tip growth, these constants can help demonstrate the requirement for active transport in polarized cell growth.

To obtain a diffusion constant from FRAP, a model must be used. The two most commonly cited FRAP models are by Axelrod et al. (Axelrod et al., 1976) and Soumpasis et al. (Soumpasis, 1983), and they use an analytically calculated recovery profile of a two-dimensional ROI inside a cell with an infinite boundary. Although these models are frequently used—over 650 combined citations to date—more recent studies have included and explored additional relevant FRAP parameters such as the finite confocal scan rate during photobleaching (Braeckmans et al., 2003; Kang et al., 2009; Deschout et al., 2010; Klassen et al.,
2010; Smisdom et al., 2011; Kang et al., 2012), arbitrary photobleaching profiles (Blumenthal et al., 2015), confocal imaging (Smisdom et al., 2011), and cell shape (Loew and Schaff, 2001; Sbalzarini et al., 2005; Mai et al., 2013). Despite this wealth of analytical models, as well as algorithmic approaches (Loew and Schaff, 2001; Sniekers and van Donkelaar, 2005; Waharte et al., 2005; Jonasson et al., 2010; Vinnakota et al., 2010; Gonzalez-Perez et al., 2011; Blumenthal et al., 2015), it remains unclear whether these models can yield accurate estimates of the diffusion coefficients for instance, when applied using realistic optical settings in tip-growing cells.

Given that understanding protein dynamics at the growing cell edge is essential to gaining a more mechanistic understanding of tip growth, there is a need for a rigorous approach that accurately measures diffusion coefficients at the cell tip. Here, we developed an experimentally validated particle-based diffusion model that allows us to conduct not only FRAP but also other fluorescence fluctuation based analyses at the cell tip.

2.3 Results

2.3.1 Analytical Modeling Demonstrates the Need to Model Cell Shape
To illustrate how the shape of the cell tip can influence FRAP based diffusion measurements, we utilized the existing one-dimensional strip FRAP model (Phillips, 2013). This model is reasonable for long tip-growing cells in which the bulk of the fluorescence recovery happens along one-dimension. The functional form for a bounded one dimensional FRAP is (Phillips, 2013),
The bleach is instantaneous, of the length 2a, and centered in the middle of a cell of the length 2L (Figure 2.1A). Here D is the diffusion coefficient of the protein of interest. However, if the bleach zone is moved to the tip of the cell (Figure 2.1B)—as would be necessary to measure protein dynamics during tip growth—the presence of the boundary should slow the fluorescence recovery. Due to symmetry, the bleach at the cell tip (as shown in Figure 2.1B) is equivalent to a centered bleach size of 4a in a cell of length 4L (as shown in Figure 2.1C).

Substitution of 2L and 2a into Eq. 3.1 indicates that the expression containing the diffusion coefficient changes from \((Dn^2\pi^2/L^2)\) to \((Dn^2\pi^2/4L^2)\). This demonstrates that the recovery at the boundary (as shown in Figure 2.1D)
recover like the centered solution (as shown in Figure. 2.1D) with an effective diffusion coefficient \( D_{eff} \equiv \frac{D}{4} \). This example quantifies the potential error an unbounded model could make if FRAP takes place close to a boundary.

Although the one-dimensional model incorporates boundaries, it assumes that the fluorescence recovery only happens in one-dimension. Because of the size of tip-growing cells, and the finite scan rate of the confocal microscope, it is not possible in most experimental setups to generate a bleach event large enough to match the idealized one-dimensional strip FRAP. Thus smaller ROIs that allow for recovery along other spatial dimensions are often used. The general solution for a square bleach in an infinite domain takes the form,

\[
FRAP_d(t) = 1 - \left[ \operatorname{erf} \left( \frac{a}{\sqrt{D} t} \right) - \frac{1}{\sqrt{\pi}} \left( 1 - e^{-\left( \frac{a^2}{D} \right)} \right) \frac{\sqrt{D t}}{a} \right]^d.
\] (Eq. 2.2)
Here the bleach is considered to be instantaneous with sides of length $2a$, $D$ is the diffusion coefficient, and $d$ is the dimensionality of the bleach (see Methods for full derivation (Kreyszig, 2006)). Because there is a greater flux when there are more spatial dimensions, the overall fluorescence recovery is faster in higher dimensions even when the diffusion coefficient is the same, as shown in Figure 2.2. If the one-dimensional model was used ($d=1$) to fit curves produced by the two and three-dimensional models ($d=2$ and $d=3$), it would predict diffusion coefficients larger by a factor of ~9 and ~29, respectively. Taken together, it is clear that the boundaries at the cell tip and the appropriate dimensionality must be taken into account to properly measure the diffusion coefficients at the cell tip.

![Figure 2.2](image)

**Figure 2.2.** Analytical demonstration of the impact of dimensionality on fluorescence recoveries. Fluorescence recoveries are predicted by Eq. 2.2 in one (blue), two (red), or three (green) dimensions. ROI is 4um in diameter, and the diffusion coefficient is $1\mu m^2/s$ for all curves.

### 2.3.2 Modeling FRAP with a Digital Confocal Microscope

Since there are no analytical models that can account for the shape of tip growing cells, we created a particle-based simulation that consists of non-interacting Brownian particles, with a constant diffusion coefficient. To mimic the geometry of
tip-growing cells, particles are contained inside a cylinder with a hemispherical cap with reflective boundary conditions.

Since small proteins such as 3mEGPF can diffuse at rates on the same order as confocal scanning, we simulated both the optics and scanning nature of the confocal microscope. Briefly, imaging is performed by scanning across the region corresponding to the output image, using a squared Gaussian beam Point Spread Function (PSF). The experimental PSF is measured with fluorescent beads (see Methods) and used to determine the parameters of the squared Gaussian. Photobleaching is performed by scanning across the ROI, and stochastically photobleaching fluorophores within the beam (see Methods). Once all the

Figure 2.3. Modeling fluorescence recoveries in tip-growing cell geometry. Three- and two-dimensional rendering of simulated scanning confocal photobleaching and recovery. Artificially fast imaging scan rates were used to illustrate three-dimensional properties of the simulation. Rainbow lookup table is used where warm colors indicate high fluorescence intensities and cool colors indicate low intensities.
aforementioned properties are incorporated, resultant simulations similar to Figure 2.3 can be generated.

2.3.3 Simulated Fluorescence Recovery is reduced at the Cell Tip

With the constructed digital confocal microscope, we sought to quantify the exact cell shape effects at the cell tip. To this aim, we simulated fluorescence recovery at different bleaching locations across the cell. We then performed fits with the one-dimensional strip FRAP model with a centered bleach. A diffusion coefficient of 1 $\mu m^2/s$ and a 4 $\mu m$ diameter ROI were used in the simulations (see Methods for more detailed parameter list). Consistent with strong boundary effects, as the bleaching event (ROI) was moved further from the cell edge, we observed an increased rate of fluorescence recovery (see Figure 2.4A). Because the one-dimensional strip model could not properly match the dimensionality of the recovery, it overestimated the fluorescence recovery when the ROI was furthest from the cell tip (see Figure 2.4B). Coincidently, the model almost predicts the correct the diffusion coefficient at the cell tip. This is because the dimensionality and boundary effects cancel, but does not indicate the strip model is accurate.

To use a model that could more accurately represent the dimensionality of the fluorescence recovery and the shape of the bleach profile, we used the model from Soumpasis et al. (Soumpasis, 1983), i.e.,

$$FRAP(t) = e^{-2\tau_D/t} \left( I_0 \left( \frac{2\tau_D}{t} \right) + I_1 \left( \frac{2\tau_D}{t} \right) \right)$$  \hspace{1cm} (Eq. 2.3)

$$\tau_D \equiv \frac{r_b^2}{4D}$$
This model uses a circular bleach region and considers the fluorescence recovery to take place in an infinite two-dimensional plane. Here $r_b$ is the radius of the bleach profile, $D$ is the diffusion coefficient, and $I_0, I_1$ are Modified Bessel functions of the First Kind. The model from Soumpasis was able to predict the correct diffusion coefficient once the bleach region was moved 6 $\mu$m from the cell edge (Figure 2.4C). This suggests that as long as the region of interest is moved one full ROI distance away from the cell edge, infinite boundary models can be used to measure diffusion coefficients.

**Figure 2.4.** ROI positional dependence. A) Simulated fluorescence recovery for an array of photobleaching regions. Color of the curve denotes position of bleach as depicted. B-C) Best-fit diffusion coefficients for the simulated recoveries in (A) when fit to the one-dimensional strip FRAP model Eq. 2.1(B) or the Soumpasis model Eq. 2.3 (C). Smaller dots are intermediate points, not depicted in (A). Note that the y-axis in (B) and (C) have different scales.
2.3.4 Cell Shape Influences the Rate of Fluorescence Recoveries in P. patens

To verify our modeling of shape effects, we performed photobleaching experiments with unconjugated 3mEGFP in vivo. We conducted these experiments in the moss Physcomitrella patens (Cove et al., 2009; Vidali and Bezanilla, 2012), at two different ROI locations, one at the cell tip and another at the center of the cell (as depicted in Figure 2.5A). We processed the curves to correct for acquisition and reversible photobleaching, and ensured that our experiments were in the linear range of the detector (see Methods). Recoveries at the cell edge exhibited a slower rate of recovery when compared to the center. To ensure that these observations were not due to filamentous actin localization at the cell edge, we performed the same analysis in the presence of an actin depolymerization agent, latrunculin B at 10μM. Latrunculin B treatment, which has been shown to completely depolymerize the actin cytoskeleton at this concentration (Vidali et al., 2009), had little influence

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**Figure 2.5.** Cell shape influences fluorescence recovery in vivo. A) Photobleaching experiments of untreated cells expressing 3mEGFP with recoveries at the edge (black squares) and center (green circles). Best fit simulation indicated in red. n=14 and 7 for the edge and center, respectively. B) Photobleaching experiments of Latrunculin B treated cells expressing 3mEGFP with recoveries at the edge (black squares) and center (green circles). Best fit simulation indicated in red. n=24 and 7 for the edge and center, respectively. Error bars represent standard deviation.
on the observed fluorescence recovery (Figure. 2.5B).

To determine if a single diffusion coefficient could reproduce the different rates of recovery observed at the edge and the center, we simulated FRAP recovery curves at the edge and center for a range of diffusion coefficients using our digital confocal microscope simulation (see Methods). In our fitting routine (see Methods), we used one diffusion coefficient to fit fluorescence recoveries at both the edge and the center, which yielded a value of $D = 8.25 \pm 0.358 \, \mu m^2/s$ (Figure 2.5A). Latrunculin treated recoveries were best fit by a similar diffusion coefficient, $D = 8.00 \pm 0.45 \, \mu m^2/s$, which is within the error of the untreated measurement (Figure 2.5B). These measured diffusion coefficients are within the expected range for a protein of this size under physiological conditions, which typically ranges between 5 and 30 $\mu m^2/s$ (Lubyphelps et al., 1985; Swaminathan et al., 1997; Elowitz et al., 1999; Hinow et al., 2006; Abu-Arish et al., 2010; Stasevich et al., 2010; Friend et al., 2017). The Einstein-Stokes relation can then be used to estimate an effective viscosity of the moss cytoplasm (Seetapun et al., 2012),

$$D = \frac{k_B T}{6 \pi \eta r} \quad \text{(Eq. 2.4)}$$

Here $k_B$ is Boltzmann’s constant, $T$ is temperature, $\eta$ is viscosity, and $r$ is the hydrodynamic radius of 3mEGFP. Using this relation, we find that the cytoplasmic viscosity is almost one order of magnitude more viscous than water (~6 fold greater).
2.3.5 Cell Shape Influences the Direction of Fluorescence Recovery *in vivo*

If cell shape influences fluorescence recoveries, intensity gradients consistent with spatial effects should be observed during the fluorescence recovery. To characterize intensity gradients during fluorescence recovery, we cropped and averaged the photobleached ROIs (Figure 2.6 and Methods section 2.5.10). We could then detect intensity gradients as a function of angular position and time within the photobleached region (Figure 6). The shape of these gradients at early time points \( \Delta t_1 = 0 - 0.1 \text{ sec} \) demonstrates that the geometry at the cell edge limited the direction of fluorescence recovery (Figure 2.6). The same analysis at the cell center yielded no observable gradient at early times \( \Delta t_1 = 0 - 0.1 \text{ sec} \) (Figure 2.8). Additionally, the uniformity of fluorescence recovery at the cell center indicates that hydrodynamic effects do not influence 3mEGPF dynamics (Figure 2.8). This also indicates that the FRAP recovery at the edge is affected by its position next to the apical plasma membrane, such that material cannot flow in or

**Figure 2.6.** Spatial fluorescence recovery of 3mEGFP. Cropped and frame averaged photobleaching ROI at the cell edge of 3mEGPF cell line (top) and simulation (bottom). Time intervals for frame averaging are \( \Delta t_1 = 0 - 0.11 \text{ s} \) and \( \Delta t_2 = 20 - 40 \text{ s} \). \( n = 14 \) and 50, respectively. ROI is 4 \( \mu m \) in diameter. Image intensity denoted with rainbow lookup table.
exchange in all directions as it can with the FRAP region in the center.

To quantitatively express the gradient’s transition from the transient state ($\Delta t_1 = 0-0.1 \text{ sec}$), found at early time points during recovery, to the steady state ($\Delta t_2 = 20-40 \text{ sec}$), and to test the statistical significance of the difference between the observed gradients, we used a Fourier cosine series to express the angular intensity profile at the cell tip (Elowitz et al., 1999)(See Methods). Over the time course of the fluorescence recovery, the coefficients of the first fundamental mode of the Fourier series, $a_1(t)$ decayed to zero (Figure 2.7E). At early times, $\Delta t_1$, the gradients exhibited time averaged Fourier coefficients significantly different than those observed during the steady state, $\Delta t_2$, (Figure 2.7F). This significant difference between the time-averaged coefficients at $\Delta t_1$ and $\Delta t_2$ indicates that experimental noise artifacts did not produce the gradient observed at $\Delta t_1$, and is consistent with a cell boundary effect on diffusion. The simulations at the cell edge recapitulated the angular intensity profiles observed transiently ($\Delta t_1 = 0 - 0.1 \text{ s}$) and at steady state ($\Delta t_2 = 20 - 40 \text{ s}$) (Figure 2.7C – 2.7F). This agreement between the simulated and experimental recovery supports the accuracy of our model of 3mEGPF diffusion, and shows that spatial effects dominate over reversible photoswitching for this system.
Figure 2.7. 3mEGPF Fourier analysis at the cell edge. A-B) Angular intensity profile of 3mEGPF at the cell edge during the time intervals $\Delta t_1$ and $\Delta t_2$. $\Delta t_1 = 0-0.11$ s (A) and $\Delta t_2 = 20-40$s (B). First mode of the Fourier series, $f_1$, is indicated in blue. n = 14, error bars indicate standard error. C-D) Angular intensity profiles of 3mEGPF simulations at the cell edge during the same time intervals. $\Delta t_1 = 0-0.1$s (C) and $\Delta t_2 = 20-40$s (D). First mode of the Fourier series, $f_1$, is indicated in blue. n=50, error bars indicate standard error. E) Amplitude of the first mode of Fourier series, $a_1$, across time for 3mEGPF (black) and simulation (red) for angular intensity profiles at the cell edge. n = 14 and 50 respectively; error bars indicate standard error. $\Delta t_1 = 0.11$s; $\Delta t_2 = 20-40$s. F) Fourier coefficients, $a_1$, for frame averaged angular intensity profiles. 3mEGPF cells are indicated in black and the simulations are indicated in red. Differences between $\Delta t_1$ and $\Delta t_2$ for simulation and 3mEGPF are significant with, p-value < 0.05 and p-value < 0.001, respectively. n = 14 and 50, for experiment and simulation, respectively; error bars indicate standard error.
2.4 Discussion

Using analytical modeling, computer simulations, and experimental approaches we demonstrate that cell shape must be taken into consideration when performing FRAP at the edge of tip-growing cells. Using two different analytical FRAP models we demonstrate that cell shape effects can significantly influence the rate of fluorescence recoveries. To account for the exact shape of tip-growing cells, we

Figure 2.8. Spatial analysis of 3mEGPF recovery at the center. A-B) 3mEGPF spatial intensity profiles at 0.11s (A) and at 20 – 40s (B) following photobleaching. Black squares represent experimental intensity, and blue line indicates first mode \( f_1 \) of the Fourier series. \( I_1(\theta) \) is the mean intensity within the sliding window for a particular angle \( \theta \). C) Amplitude of the first mode of Fourier series across time, \( a_1 \). D) Binned first mode, \( a_1 \), values at 0.11s and 20 – 40s. Error bars represent standard errors. p-value = 0.28 (n=7)
constructed a three-dimensional particle based FRAP simulation. We then compared the fluorescence recoveries predicted by our computational model and the two existing analytical models. We demonstrated that the one-dimensional strip FRAP model could overestimate diffusion coefficients by a factor of five in the center of tip growing cells. This was largely the case because it failed to match the dimensionality of the fluorescence recovery. We further showed that the Soumpasis model, which incorporates two-dimensional recoveries in an infinite domain, could underestimate diffusion coefficients by a factor of more than two at the cell tip. These shape effects have important implications with regards to cell signaling and cell growth (Rangamani et al., 2013). For example, diffusing proteins concentrated to the cell tip will reside at the cell tip longer than diffusing proteins concentrated at the cell shank.

Through experimental FRAP of unconjugated 3mEGFP, we were able to demonstrate the expected shape effects in vivo. We also measured the diffusion coefficient of 3mEGFP to be $8.25 \, \mu m^2/s$ and the viscosity of the moss cytoplasm to be 6.3 times more viscous than water. These kinds of measurements are important because they provide insight into all facets of cellular dynamics, which influence fundamental cellular processes such as growth, division, and cell signaling. Additionally, this viscosity measurement can be used to estimate diffusion of other proteins.
2.5 Methods

2.5.1 Experiment Imaging
FRAP experiments were conducted using a Leica TCS SP5 scanning confocal microscope and the Leica FRAP Wizard. To conduct FRAP experiments, a 63X objective was used with a numerical aperture (NA) of 1.4. In the software settings, the pinhole was set to 2.00 airy disks and the camera zoom was set to 9. Images of 256×256 pixels were acquired with a depth of 12bits. To visualize 3mEGPF, the Argon laser was set to 75% power with a bidirectional scanning speed of 2800 Hz and the 488 nm laser line was set to 10% power in the FRAP wizard. The emission bandwidth for 3mEGPF was set between 499 nm and 546 nm. During bleaching events, all laser lines were set to 100%.

2.5.2 Experimental Cell Culture and Sample Preparation
FRAP experiments were conducted on the caulonemal cells of the moss Physcomitrella patens; moss tissue was cultured on cellophane placed on top of the solidified agar. Microscope samples were prepared in QR-43C chambers (Warner Instruments) as follows. First, 25 mm bottom coverslips were plasma treated for three minutes to yield a hydrophilic surface. A solution of 0.8% type VII agarose in PpNO3 medium (refer to (Furt et al., 2013) for details) was melted then added directly to the coverslips. A small cellophane square (1 cm²) with moss tissue, grown for seven days after sub-culturing, was inverted and placed onto the agarose. To obtain flat cultures, a second untreated coverslip was placed on top of the cellophane and flattened using a blunted syringe. Agarose was solidified by placing the cultures onto a surface at 12°C. Once the agarose solidified, the top
coverslip was gently removed from the top of the preparation. The remaining preparation was submerged in PpNO3 and the cellophane was removed. With the moss firmly adhered to the agarose, the entire coverslip was added to the QR chamber. The chambers were capped with 18 mm coverslips and connected to silicone tubing with inner and outer diameters of 0.03 in and 0.065 in, respectively. The tubing was connected to a peristaltic pump and liquid PpNO3 was perfused through the chambers overnight. Liquid PpNO3 was made two days prior to perfusion and was filter sterilized immediately before its use. During latrunculin B treatment, a solution of 10 μM latrunculin B in PpNO3 was perfused through the chamber for 20 minutes.

2.5.3 Modeling Diffusion
We used a particle-based approach to simulate diffusion during FRAP. Assuming that the moss cytoplasm is a fluid with a low Reynolds number, the displacement for a given protein along any one direction can be simulated by sampling from a Gaussian distribution with a mean of zero and a variance equal to 2Dt (Arpag et al., 2014; Machan et al., 2016). Here t is the time step of the simulation. Reflective boundary conditions were used to ensure that the proteins remained inside the cell boundary.

2.5.4 Modeling and Measuring Confocal Imaging
The point spread function (PSF) of a confocal microscope is the diffraction pattern produced by a single point source of light. To simulate imaging, the PSF is convolved with the proteins in the field of view. In order to appropriately model confocal imaging, a function must be chosen that reasonably approximates the
experimental PSF. To this aim we found that a squared Gaussian beam well approximates our experimental imaging PSF (Figure 2.9),

\[ I_{PSF}^{(x,y,z)} = \left( \frac{l_0}{w(x,z)} \right)^2 e^{-\frac{4(x^2+y^2)}{w^2(x,z)}} \]  
\[ (Eq. 2.5) \]

\[ w(z) = w_0 \sqrt{1 + \frac{z^2}{z_R}} \]  
\[ (Eq. 2.6) \]

This function is a better fit to our experimental PSF when compared to the traditionally used 3D Gaussian function (Figure 2.9). The open parameters for the Gaussian beam Eqs. 2.5 and 2.6 were \( w_0 \) and \( z_R \), and were found to be \( 350 \pm 25 \) and \( 875 \pm 25 \) nm, respectively. The 3D Gaussian is of the form,

\[ I_{PSF} = l_0 e^{-2(x^2+y^2+z^2/\kappa^2)/w^2}. \]  
\[ (Eq. 2.7) \]

The open parameters were \( w \) and \( \kappa \).

To experimentally measure the PSF of the confocal microscope, beads were used from the Invitrogen PS-Speck Microscope Point Source Kit P7220 (Thermo Fisher Scientific, Waltham, MA). Preparations were made by adding 5 μL of 0.01% polylysine and 5 μL of bead solution to a dry microscope slide. A coverslip was then placed directly onto the slide and sealed with wax. Beads were visualized and measured with the Leica TCS SP5 scanning confocal microscope, using the settings described above. Green fluorescent beads were used to match the 3mEGPF fluorophore, and z-stacks of the 175 nm beads were taken. From these z-stacks, a three-dimensional reconstruction of the bead’s intensity profile was
created as shown in Figure. 2.9A. These profiles were then used to determine the three-dimensional functional form and parameters of the PSF.

2.5.5 Modeling and Measuring Confocal Bleaching

During photobleaching, the confocal scans a high-intensity laser across the photobleaching ROI to photobleach the fluorophore of interest. In the simulation, the ROI is bleached with successive bleach line scans that together construct the

Figure 2.9. Measuring the imaging Point Spread Function (PSF). A) Squared Gaussian Beam approximation \( I^{PSF}(x,y,z) \) (left, defined by Eq. 3.7) of experimental point spread function for 3mEGPF (right). B) Parameter scan of potential values for \( w_0 \) and \( z_R \). Cool colors indicate small sum of squares between the experimental PSF and Squared Gaussian Beam, while warm colors indicate high sum of squares. C) Sum of squares between the experimental PSF and either the Squared Gaussian Beam (red) or the 3D Gaussian (blue).
circular ROI. The simulation scan time between each line is set by the experimental scan time, 2800Hz. Each bleach line is represented by a Gaussian beam convolved with a boxcar function from \( x = -a \) to \( x = a \), i.e.,

\[
P_{\text{photobleach}}(x, y, z) = K \int_{-a}^{a} I(x + u, y, z) du = \int_{-a}^{a} e^{-\frac{2((u+x)^2+y^2)}{w^2(z)}} du
\]

\[
= \frac{K}{w(z)} \left[ \text{erf} \left( \frac{\sqrt{2}(x+a)}{w(z)} \right) - \text{erf} \left( \frac{\sqrt{2}(x-a)}{w(z)} \right) \right] \times \left[ e^{-\frac{2y^2}{w^2(z)}} \right]. \quad \text{(Eq. 2.8)}
\]

Here \( K \) is the proportionality constant that controls the maximum probability of bleaching a fluorophore in the center of the bleach line. \( K \) was left as an open parameter during fitting to experimental fluorescence recoveries.

### 2.5.6 Image Acquisition Bleaching and Reversible Photobleaching

Image acquisition photobleaching and reversible photoswitching during imaging, are processes by which fluorophores are photobleached or photo-converted by the laser used to excite the fluorophores. Depending on the experimental setup and conditions, such as the choice of fluorophore of interest and laser intensity, image acquisition photobleaching can significantly reduce fluorescence recoveries and lead to underestimation of the diffusion coefficient (Sinnecker et al., 2005; Mueller et al., 2012; Morisaki and McNally, 2014).

To measure the contribution of these effects, fluorescent cells were imaged with the same image sampling rate and experimental conditions as those used during FRAP experiments (see Experimental Imaging). Cells were not subjected to the high-intensity laser pulse necessary during FRAP experiments. Thus,
reduced fluorescent intensity was primarily a result of the photobleaching and photoswitching during image acquisition. Images were then analyzed, and the mean fluorescence loss within the ROIs at the cell edge and center were measured across time. This ensured that the appropriate boundary effects were taken into consideration for each location. Fluorescence loss $R_L(t)_i$ was divided by the intensity measured within the ROIs for the first image acquired $R_L(0)_i$ to yield $R_{Ln}(t)_i$. Here, the subscript $i$ denotes the $i$th experimental replicate. These normalized curves $R_{Ln}(t)_i$ were averaged across these replicates $< R_{Ln}(t)_i >_i$ to yield $R_{Ln}(t)$. Examples of these curves from the cell center of latrunculin B treated cells expressing 3mEGPF are displayed in Figure 2.10A.

The observed intensity profile shows a short bleaching event, followed by two longer recovery events at the input acquisition rates. This initial decay was less than 2% because we tried to minimize the laser intensity (10% power) required to illuminate our sample. Since our pinhole size was 2 Airy Units, we could collect sufficient light at low laser power. To help prevent further fluorescence loss, we then changed our sampling rate after the first 30 points were selected. This decrease in imaging sampling rate caused a small increase in fluorescence intensity, an effect which has previously been shown to be the product of reversible photoswitching during image acquisition (Sinnecker et al., 2005; Mueller et al., 2012; Morisaki and McNally, 2014). Furthermore, this type of reversible photobleaching cannot be explained by a single light dependent rate constant that converts unbleached fluorophores to permanently bleached ones.
As a first approximation to this reversible photoswitching process, one would have to determine the rate constants that dictate the reversible bleaching reaction \( PB \leftarrow F \rightleftharpoons RB \), where PB, F, and RB are the concentrations of permanently bleached, unbleached, and reversibly photoswitched fluorophores, respectively. However, this reaction can include additional states and transitions (such as from RB to PB), and the relevant rate constants have been shown to be light dependent (Sinnecker et al., 2005), increasing the number of open parameters in the model that need to be experimentally determined. While DCMS is capable of reproducing this reversible photoswitching, to avoid a large reaction model and parameter scan, and increase the accuracy of our approach, we sought to remove acquisition photobleaching/reversible photoswitching effects from our experimental data directly (Phair et al., 2004).

Since these \( R_{Ln}(t) \) curves were noisy, they were approximated by separate exponential functions as shown in Figure 2.10A. Specifically, the three image sampling rates of 0.11, 0.25, and 1 s/frame used throughout the time course were each fit to their own exponential function, resulting in one curve, \( R(t) \), that approximates the entire image acquisition time course \( R_{Ln}(t) \), i.e.,

\[
R(t) = \begin{cases} 
A_1 e^{B_2 t} + C_1 & \text{for } 0 < t \leq 3.39 \\
A_1 e^{B_2 t} + C_1 & \text{for } 3.39 < t \leq 10.89 \\
A_1 e^{B_2 t} + C_1 & \text{for } 10.89 < t \leq 40 \
\end{cases}
\]  
(Eq. 2.9)
Finally, to correct for image acquisition photobleaching/reversible photoswitching each averaged FRAP experiment $< R^{exp}(t) >$ was divided by its corresponding acquisition approximation $R(t)$ to yield $R^{exp}(t)$. Examples of fluorescent recovery curves before $< R^{exp}(t) >$ and after acquisition bleaching/reversible photoswitching correction $R^{exp}(t)$ can be found in Figure 2.10B. Following correction, these recovery curves more accurately depict the dynamic nature of the fluorophores in question without being confounded by image acquisition photobleaching or reversible photoswitching, and can now be analyzed with a FRAP model that does not incorporate these effects.

**Figure 2.10.** Image acquisition photobleaching and reversible photoswitching. A) Observed photobleaching and photoswitching during image acquisition at the center of cells expressing 3mEGPF treated with latrunculin B. Red squares represent mean acquisition intensity, $R_{ln}(t)$. Regions of different image sampling rates are outlined above. Blue dashed lines represents piecewise exponential approximation of image acquisition effects $R(t)$, see Eq. 2.9. Error bars represent standard error. (n=9). B) Example fluorescence recovery before $< R^{exp}(t) >$ (black triangles) and after $R^{exp}(t)$ (red squares) correction for acquisition photobleaching and reversible photoswitching. Correction was conducted by dividing $< R^{exp}(t) >$ by the acquisition correction $R(t)$ (blue) for the cell center of latrunculin B treated cells expressing 3mEGPF. n=13 and 6, respectively.
2.5.7 Bleaching and Reversible Photobleaching

Similar to reversible photoswitching effects during acquisition, one should expect the main photobleaching event to be influenced by the photoconversion of the fluorescent protein (Sinnecker et al., 2005; Mueller et al., 2012). To measure the effects of reversible photobleaching, we performed photobleaching experiments in the nucleus of a cell line expressing nuclear GFP. During photobleaching, we illuminated the entire nucleus in plane, eliminating the effects of fluorescence recovery in the x and y directions. Based on the size of our PSF and our simulation evidence, fluorescence recovery in the z direction can be neglected. For these reasons, we attribute the fluorescence recovery seen in the nucleus to reversible photoswitching (see Figure 2.11A). We found that the proportion of reversibly photoswitched molecules, $\alpha$, was approximately 11%. To approximate the rate of reversible photoswitching, $R(t)$, the fluorescence recovery was fit to a double exponential. As shown previously, (Mueller et al., 2012), we found that $\alpha$ increased as we decreased the laser power of the main bleach event (Figure 2.11B).

While our digital confocal simulation can model this reversible photoswitching during bleaching, once again, for similar reasons, we opted to use an empirical approach and corrected for reversible photoswitching using a scheme devised by Mueller et al. (Mueller et al., 2012). Specifically, we modified all the simulation outputs with the correction scheme (Mueller et al., 2012) expressed as,

$$I_M(r,t) = I_{FRAP}(r,t) + \alpha R(t)I_{FLAP}(r,t).$$  \hfill (Eq 2.10)
Here $I_M(r,t)$ represents the observed fluorescence recovery as a function of space and time for a photobleaching experiment with irreversible photoswitching, $I_{FRAP}(r,t)$ represents the contribution of fluorescence recovery from unbleached

### Figure 2.11

Reversible photoswitching caused by the main photobleaching event

**A)** Measured reversible photoswitching of nuclear GFP, demonstrated by $I_N(t) = \frac{\langle I_M(r,t) \rangle_{(x,y)}}{\langle I_M(r,t<0) \rangle_{(x,y)}}$. $\alpha$ denotes the percentage of reversibly switched fluorophores. $n=4$, error bars indicate standard error. Width of the nucleus images is 9 $\mu$m. **B)** Fluorescence recoveries, $I_F(t) = \frac{I_N(t) - I_N(t=0.21s)}{I_N(t<0) - I_N(t=0.21s)}$ due to reversible photoswitching of nuclear GFP at 100% (black), 50% (blue), 30% (red), and 15% (grey) laser power. **C)** Illustration of reversible photoswitching components including the rate of reactivation $R(t)$ (yellow), the rate of their movement out of the ROI $I_{FLAP}(t)$ (blue), and their product $R(t)I_{FLAP}(t)$ (red). **D)** DCMS-produced curves $FRAP(t)$ (black) before correction for reversible photoswitching, the contribution of reversible photoswitching $\alpha R(t)I_{FLAP}(t)$ (red inset), and the final corrected DCMS simulation $FRAP(t) + \alpha R(t)I_{FLAP}(t) = FRAP(t) + \alpha R(t)[1 - I_{FRAP}(r,t)]$ (blue).
fluorophores into the bleached region, and the expression $\alpha R(t)I_{FLAP}(r,t)$ is the contribution of fluorescence recovery due to reversibly photoswitched fluorophores. $I_{FLAP}(r,t)$ is used here because the population of reversibly photoswitched fluorophores can be thought of as a Fluorescence Loss After Photobleaching (FLAP) experiment. FLAP is a process by which a high-intensity laser pulse activates a population of fluorophores. The fluorescence intensity within this zone of activation will decrease as the activated fluorophores diffuse away. As shown by Mueller et al. (Mueller et al., 2012), $I_{FLAP}(r,t) = [1 - I_{FRAP}(r,t)]$ and $[1 - I_{FRAP}(r,t)]$ can be substituted into Eq. 2.10. This is true because the laser pulse in both FRAP and FLAP is the same. It then follows that the solution to the diffusion equation for FLAP is equal to one minus the FRAP solution. Since our simulation produces images with the intensity profile $I_{FRAP}(r,t)$ including all the necessary spatial effects, $I_{FLAP}(r,t)$ also includes spatial effects. Given our measured $R(t)$ and $\alpha$, we were able to correct all DCMS outputs for reversible photoswitching (including spatial effects) using this approach (Figure 2.11C and 2.11D).

### 2.5.8 FRAP Parameter Minimization

To identify the simulation parameters that best fit the recovery curves produced by experiments, a least squares minimization routine was used. In this routine, an exhaustive parameter sweep was simulated to generate a library of simulated recovery curves for all relevant parameter combinations. Then based on the quality of the fit to the experimental curves, the best-fit parameters were identified. Specifically, averaged experimental recovery curves $R^{exp}(t)$ were characterized
into classes $R_{E}^{\text{exp}}(t)$ and $R_{C}^{\text{exp}}(t)$, depending on if they were produced from the edge or center of the cell, respectively. Similarly, the averaged simulation recovery curves were characterized to produce $R_{E}^{\text{sim}}(t)_{D,K,w_{o}}$ and $R_{C}^{\text{sim}}(t)_{D,K,w_{o}}$. Here, $D$ is the diffusion coefficient, $K$ is the bleaching proportionality coefficient, and $w_{o}$ is the bleaching probability beam waist used of the Gaussian beam in the simulation. The best fit parameters for each experimental condition were found by minimizing the sum of squares differences between the experiment and simulation, for both the edge and center i.e.,

$$\text{argmin}_{w_{o},D,K_{E},K_{C}}\left[\sum_{t>0}(R_{E}^{\text{exp}}(t) - R_{E}^{\text{sim}}(t)_{D,K_{E},w_{o}})^{2} + \sum_{t>0}(R_{C}^{\text{exp}}(t) - R_{C}^{\text{sim}}(t)_{D,K_{C},w_{o}})^{2}\right]$$

(Eq. 2.11)

These parameters were found by allowing the edge and center to have the same diffusion coefficient $D$. The bleaching proportionality coefficient, $K$, was split into two independent parameters $K_{E}$ and $K_{C}$, for the edge and center, respectively, to improve the resultant fit. The difference between $K_{E}$ and $K_{C}$ indicates that some underlying physical process may be altering the initial bleach depths at the edge and center (Table 2.1). Understanding the cause of this difference is beyond the scope of this work. Note that this parameter minimization procedure was only applied to experimental data, using the digital confocal simulation generated recovery curves. Errors associated with the parameters were determined from the minimization procedure described in the following section 3.5.9.

2.5.9 FRAP Parameter Error Estimation

In order to provide a confidence interval on the parameters determined from the
parameter minimization procedure, we used a Monte Carlo method. In the Monte Carlo method used, we took the mean and standard deviation for the average recovery curves, $R^{exp}(t)$ and $\sigma^{exp}(t)$, respectively, and generated a series of new recovery curves, $\gamma(t)_i$, from them. This was done by sampling each point from a normal distribution with the parameters given by the experimental data, i.e.,

$$\gamma^{exp}(t)_i = N(R^{exp}(t), \sigma^{exp}(t)).$$  \hspace{1cm} (Eq. 2.12)

Here $i$ represents the $i^{th}$ generated recovery curve. This generation process was repeated $n$ times, where $n$ matches that of the experiment, and was then averaged to produce $\gamma^{exp}(t)$. A similar procedure was then repeated on the simulation data (where $n = 50$), for all of the varying parameters, producing a new library of $\gamma^{sim}(t)_{D,K,w_0}$. The minimization procedure was then applied to find the $D, K_C, K_E$, and $w_0$ for that set, as in Eq. 2.11,

$$\arg \min_{w_0,D,K,C,K_E} \left[ \sum_{t > 0} (\gamma^{exp}_E(t) - \gamma^{sim}_{D,K,C,w_0}(t))^2 + \sum_{t > 0} (\gamma^{exp}_C(t) - \gamma^{sim}_{D,K,C,w_0}(t))^2 \right].$$  \hspace{1cm} (Eq. 2.13)

The random resampling imposed by both $\gamma^{exp}(t)$ and $\gamma^{sim}(t)$ allows for variation of the minimized parameters $D, K_C, K_E$, and $w_0$ each time this routine is repeated. We then repeated this procedure until the standard deviation of the parameters $D, K_C, K_E$, and $w_0$ reaches convergence. The confidence of these parameters was then expressed as two standard deviations of each parameter, as found in Table 3.1. Once again, this procedure was only applied to the experimental data, based on the DCMS-produced curves.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imaging $w_0$</td>
<td>$350 \pm 25$ nm</td>
</tr>
<tr>
<td>Imaging $z_R$</td>
<td>$875 \pm 25$ nm</td>
</tr>
<tr>
<td>Bleaching $w_0$</td>
<td>$950 \pm 158$ nm</td>
</tr>
<tr>
<td>Bleaching $K_E$</td>
<td>$0.006 \pm 0.0015$</td>
</tr>
<tr>
<td>Bleaching $K_c$</td>
<td>$0.008 \pm 0.0015$</td>
</tr>
</tbody>
</table>

Table 2.1. FRAP parameters determined for 3xmEGFP. Imaging parameters include the beam waist, $w_0$ and Rayleigh range, $z_R$. Confidence intervals are defined by the sampling distance for the respective parameter, as illustrated by the grid in Figure 9. Photobleaching parameters include the beam waist, $w_0$, and bleaching probabilities, $K_E$ and $K_c$, for the edge and center, respectively with 95% confidence intervals. $K_E$ and $K_c$ Monte Carlo replicas never produced different parameters. For this reason, the confidence intervals for these parameters are represented by our simulation sampling distance for the parameter $K$. It is important to note that using a shared bleach probability for the edge and center did not appreciably change our measured diffusion coefficient.

### 2.5.10 Spatial Analysis of Fluorescence Recovery

To enable spatial analysis of fluorescence recoveries, experimental bleaching ROIs were extracted from image stacks with a custom cropping macro written in ImageJ. During cropping, the images were manually oriented and the macro was used to crop the ROIs from each image stack. Cropped image stacks were divided by the mean of their corresponding prebleach images and then averaged across replicate experiments, namely,

$$I_n(x, y, t)_i = \frac{I(x, y, t)_i}{\langle I(x, y, t)_{i(x, y, t<0)} \rangle}.$$  \hspace{1cm} (Eq. 2.14)

Here $I(x, y, t)_i$ is the $i^{th}$ image stack for a particular experimental condition, $t < 0$ denotes the prebleach portion of time in the image stack, and $I_n(x, y, t)_i$ is the final normalized image stack. Simulated images were cropped and divided by the mean prebleach intensity in the same way.
To remove the effects of medial plane deviation and the limited accessible volume at the cell edge, described in (Methods Effects and Photobleaching Away from the Medial Cell Plane), the extracted ROIs were again normalized. To conduct this second normalization, prebleach ROIs were averaged in time and along the vertical axis of the cell, as shown in Figure 2.12. Zero valued pixels were ignored in this averaging. More specifically,

\[ I_{Line}(x) = \langle I_n(x, y, t)_i \rangle_{y,t<0} \quad \text{if} \quad I_n(x, y, t)_i > 0 \quad \text{(Eq. 2.15)} \]

These line scans \( I_{line}(x) \) were fit to an exponential function of the form \( Ae^{-Bx} + C \). Each ROI stack \( I_n(x, y, t)_i \) was then divided by this exponential decay to yield a volume corrected ROI \( I_v(x, y, t)_i \), as shown in Figure 2.12, i.e.,

\[ I_v(x, y, t)_i = \frac{I_n(x, y, t)_i}{Ae^{-Bx} + C} \quad \text{(Eq. 2.16)} \]

Volume corrected ROIs were used in all subsequent spatial analyses.

**Figure 2.12.** Accessible volume and medial plane deviation correction. A) 3mEGPF at cell edge before (left) and after (right) accessible volume correction. \( (n = 13) \) B) 3mEGPF simulation at cell edge before (left) and after (right) accessible volume correction. \( (n = 50) \) All cropped ROIs are 4 μm in diameter.
2.5.11 Fourier Analysis

Quantification of the directionality of fluorescence recovery was performed by subsampling the cropped ROIs into angular sectors about their centers. To reduce the noise associated with small regions of interest, a sliding window average was used. This sliding window was rotated 360 degrees and averaged about the horizontal axis of the ROI, producing an intensity profile that was dependent on the angular position of the sliding window, as shown in Figure 2.13. To investigate fundamental modes of spatial recovery, the angular intensity profiles, $I_v$, were reconstructed using a Fourier cosine series (Elowitz et al., 1999), namely,

$$I_v(\theta) = \sum_{n=0}^{\infty} f_n(\theta) = \frac{a_0}{2} + \sum_{n=1}^{\infty} a_n \cos(n\theta), \quad (Eq. 2.17)$$

with $a_n$ denoting the amplitude of the $n^{th}$ Fourier mode. To illustrate the trends captured by the Fourier coefficients, three artificial gradients were generated and a Fourier analysis was used. The first gradient (Figure 2.13A) increases linearly from right to left, while the second (Figure 2.13B) peaks at 120 degrees. Finally, the third gradient (Figure 2.13C) increases from right to left. The two linear gradients (Figures 2.13A and 2.13C) were well approximated by the first cosine mode of the Fourier series. This first ($n = 1$) amplitude describes the direction and steepness of the gradient. Specifically, a positive amplitude represents a gradient increasing from right to left, and a negative amplitude represents a gradient increasing from left to right. The mode amplitude value represents the steepness of the gradient. The gradient with a peak (Figure 2.13B) requires the first three Fourier modes to be best captured. These simple examples illustrate how signs and amplitudes of the first few terms of the cosine series throughout fluorescence...
recovery make it possible to quantitatively characterize the directionality of spatial recovery.

**Figure 2.13.** Fourier analysis of ROIs. A) Sliding angular intensity window for spatial analysis of generated gradients is indicated in red. Window is rotated 360 degrees and averaged about the ROIs horizontal axis. B) Fourier series representation of spatial intensity for generated gradients. Black squares represent the mean intensity of the sliding window, $I_\nu(\theta)$, divided by the maximum pixel intensity of the image, $I_{max}$. Dotted lines indicate $f_n$ where $f_0$ is red, $f_1$ is green, $f_2$ is blue, and $f_3$ is magenta (Eq. 2.17).

### 2.5.12 Detector Linearity

One of the main assumptions inherent in current analytical and computational FRAP models and analysis is detector linearity (Waters, 2009). This assumption asserts that an increase in measured fluorescence intensity is linearly proportional to the increase of fluorescent particles within a region of interest, and holds well within a limited range, dependent on experimental setup. Due to the limited range of linearity of photon detectors in confocal systems, it was necessary to establish an operating range for the FRAP experiments in our experimental setup. Operating outside this linear range can produce results that are not comparable with those within the linear regime. To establish an operating range, a cell line with nuclear localized GFP was used. Repeated photobleaching was conducted to reduce the concentration of fluorescent particles within the nucleus, as shown in Figure 2.14.
FRAP recovery curves $R_{m}^{nuc}(t)$ were generated by measuring the mean nuclear GFP intensity at each time point, $t$. Here, the subscript $m$ denotes the $m^{th}$ successive bleach and the superscript $nuc$ denotes that the intensity measured was from nuclear GFP. These curves were then divided by the nuclear GFP prebleach intensities $R_{m}^{pbnu}$ which corresponds to each successive bleach. By measuring fluorescence recovery at different concentrations we were able to determine if the concentration of particles could affect the resulting FRAP curves (Figure 2.14). Since there was no difference in FRAP profiles across experimentally relevant intensities, it was concluded that these experiments were conducted within the linear operating range of the detector. Because the detector linearity assumption holds, no confocal detector corrections were applied to experimental FRAP data.

Figure 2.14. Photon detection linearity. Nuclear GFP prior to photobleaching and following nine successive photobleaching and recovery experiments (left). Example fluorescence recovery of nuclear GFP after the first (red circles) and ninth (black triangles) successive bleaching events (right). $R_{m}^{nuc}(t)$ is the nuclear GFP fluorescent intensity and $R_{m}^{pbnu}$ is the mean prebleach nuclear GFP intensity before each successive bleach.
2.5.13 Imaging and Bleaching Away from the Medial Cell Plane

With and without treatment with latrunculin B, an intensity gradient was detectable at the edge of the cell for the 3mEGPF fluorophores, Figure 2.15A. It is possible that these gradients are partially a result of imaging above and below the medial cell plane. These intensity gradients were observed prior to photobleaching and decayed toward the edge of the cell (Figure 2.15C). Intensity gradients were quantified by taking line scans $L(x)_i$ through cells that were not subjected to photobleaching. Intensity gradients $L(x)_i$ were divided by their corresponding center intensity at $L(0)_i$ and averaged about the $i^{th}$ image, $< L(x)_i/L(0)_i >_i$.

Since these gradients were present in the 3mEGPF cell lines, they were not the result of secretion at the cell edge. To determine if this reduced edge intensity could be a result of the reduced cell volume imaged at the edge (Dalal et al., 2008), confocal images of uniformly distributed fluorophores were simulated, and the

**Figure 2.15.** Intensity profile variation in Z. A) Experimental intensity gradient measured with unconjugated 3xmEGFP. Dashed white line indicates line scan through ROI at cell edge. ROI is 4 μm in diameter. B) Simulated intensity gradients for image acquisition medially at $Z = 0$ μm and 2.5 μm above the medial plane. Dashed white lines indicate line scans through simulations. C) Horizontal line scans through simulation at $Z = 0$ μm (dashed blue line) and $Z = 2.5$ μm (dashed red line). Line scan through experimental 3xmEGFP intensity profile (black squares). $< L(x)_i/L(0)_i >_i$ denotes that the experimental line scan intensities were divided by the center intensity, and averaged across experimental replicates. Error bars indicate standard error. $n=14$ for experimental 3xmEGFP and $n=50$ for simulations.
results for two different slices in Z are shown in Figure 2.15B. In the medial plane at \( Z = 0 \), the reduced volume alone could not reproduce the experimental line scans. When imaged 2.5 \( \mu m \) above or below the medial cell plane, the simulations were able to produce a stronger gradient effect than the one observed experimentally, shown in Figure 2.15C. This suggests that the experimental gradient is a result of imaging above and below the medial plane.

It is likely that the photobleaching experiments happened around the medial plane, \( Z = 0 \), with some variation in Z positioning plus or minus a few microns. To test the potential effects of this on the rate of fluorescence recovery, FRAP experiments, with a diffusion coefficient of \( D = 0.35 \ \mu m^2 / s \) (to better resolve this effect), were simulated at the medial plane and 2 \( \mu m \) away from the medial plane, Figure 2.16A. These simulated curves were averaged about each replicate \( < R_{0}^{sim}(t) > \) to yield \( R^{sim}(t) \) for both imaging planes. Both recovery profiles for \( Z = 0 \ \mu m \) and \( Z = 2 \ \mu m \) shown in Figure 2.16B are very similar. Moreover, randomly generated simulations with a 2 \( \mu m \) standard deviation away from the medial plane were generated, as displayed in Figure 2.16C. These simulations had a constant diffusion coefficient of \( D = 0.35 \ \mu m^2 / s \) and were bound by recovery curves of simulations photobleached at the medial cell plane with diffusion coefficients \( D = 0.31 \ \mu m^2 / s \) and \( D = 0.39 \ \mu m^2 / s \). This error in diffusion coefficient measurement is well within a 15% error, as depicted in Figure 2.16C.
Based on these two analyses, it can be concluded that imaging away from the medial plane can influence the spatial FRAP analysis, but photobleaching away from the medial plane has little influence on FRAP recovery curves. As a result, only spatial FRAP analysis was subjected to corrections to account for imaging away from the medial plane.

![Figure 2.16](image)

**Figure 2.16.** Recovery dependence on photobleaching plane position in Z. A) Simulated photobleaching medially at Z = 0 μm (top) and 2 μm above the medial plane (bottom). B) Simulated fluorescence recovery for photobleaching at Z = 0 μm (dashed blue line) and Z = 2 μm (black line). $R^{sim}(t)$ is the averaged simulation recovery curve. (n=50) C) Simulated averaged fluorescence recovery for randomly chosen FRAP experiments performed between the medial plane at Z = 0 μm and 2 μm above with a diffusion coefficient of $D = 0.35 \mu m^2/s$ (blue line). Dotted lines represent simulated medial fluorescence recovery at Z = 0 μm with diffusion coefficients $D = 0.31 \mu m^2/s$ (dashed red line) and $D = 0.39 \mu m^2/s$ (dashed black line), respectively, and $R^{sim}(t)$ is the averaged simulation recovery curve. (n=50)

### 2.5.14 Derivation of FRAP Recovery in Multiple Dimensions

While the analytical calculations are straightforward in Cartesian coordinates (Kreyszig, 2006) for FRAP in an infinite domain, we outline the steps here for completeness. Starting from the three-dimensional diffusion equation,

$$\frac{\partial c(x,y,z,t)}{\partial x} = D\nabla^2 c(x, y, t, z) \quad \text{ (Eq. 2.18)}$$

with the initial condition
\[
c(x, y, z, 0) = \begin{cases} 
1 & x, y, z \in [-a, a] \\
0 & \text{else}
\end{cases} \quad (\text{Eq. 2.19})
\]

\[
c(x, y, z, 0) = \{0 \text{ else}\}, \quad (\text{Eq. 2.20})
\]

one can show the FRAP recovery is given by

\[
FRAP(t) = 1 - \frac{1}{V} \iiint_V c(x, y, z, t)dx dy dz. \quad (\text{Eq. 2.21})
\]

Substituting the Laplacian in three-dimensional Cartesian coordinates the diffusion equation becomes

\[
\frac{\partial c(x,y,z,t)}{\partial x} = D \left( \frac{\partial^2 c}{\partial x^2} + \frac{\partial^2 c}{\partial y^2} + \frac{\partial^2 c}{\partial z^2} \right). \quad (\text{Eq. 2.22})
\]

Using separation of variables the diffusion equation can be written as the product of four functions each with one variable,

\[
c(x,y,z,t) = X(x)Y(y)Z(z)F(t). \quad (\text{Eq. 2.23})
\]

Substitution of the new form of the concentration profile into the diffusion equation yields the following equation that must equal a constant,

\[
\frac{1}{DF} \frac{dF}{dt} = -\lambda^2 = \frac{1}{X} \frac{d^2X}{dx^2} + \frac{1}{Y} \frac{d^2Y}{dy^2} + \frac{1}{Z} \frac{d^2Z}{dz^2}. \quad (\text{Eq. 2.24})
\]

Assuming the concentration profile should decay over time, the solution for the time-dependent function \(F(t)\) becomes,

\[
F(t) = C_1 e^{-\lambda^2Dt}. \quad (\text{Eq. 2.25})
\]

Each term in the spatial portion of the solution must also equal a constant so it...
follows that,

\[-\frac{1}{x} \frac{d^2x}{dx} = \beta^2 = \frac{1}{y} \frac{d^2y}{dy} + \frac{1}{z} \frac{d^2z}{dz} + \lambda^2 \]  
(Eq. 2.26)

\[-\frac{1}{y} \frac{d^2y}{dy} = \eta^2 = \frac{1}{z} \frac{d^2z}{dz} - \beta^2 + \lambda^2 \]  
(Eq. 2.27)

\[\mu^2 = -\eta^2 - \beta^2 + \lambda^2. \]  
(Eq. 2.28)

Solving each separate differential equation yields the following three solutions

\[X(x) = \tilde{A}(\beta) \cos(\beta x) + \tilde{B}(\beta) \sin(\beta x) \]  
(Eq. 2.29)

\[Y(y) = \tilde{C}(\eta) \cos(\eta y) + \tilde{D}(\eta) \sin(\eta y) \]  
(Eq. 2.30)

\[Z(z) = \tilde{E}(\mu) \cos(\mu z) + \tilde{F}(\mu) \sin(\mu z) \]  
(Eq. 2.31)

One can then show that the concentration at \( t = 0 \) can be written as

\[c(x, y, z, 0) = \int_0^{\infty} d\beta \int_0^{\infty} d\eta \int_0^{\infty} d\mu \left[ \tilde{A}(\beta) \cos(\beta x) + \tilde{B}(\beta) \sin(\beta x) \right] \times \]

\[\left[ \tilde{C}(\eta) \cos(\eta y) + \tilde{D}(\eta) \sin(\eta y) \right] \times \left[ \tilde{E}(\mu) \cos(\mu z) + \tilde{F}(\mu) \sin(\mu z) \right]. \]

(Eq. 2.31)

The coefficients \( \tilde{A} \) through \( \tilde{F} \), can be determined from the initial conditions using the orthogonally relations of trigonometric functions. The general solutions can be written as
\[ c(x,y,z,t) = \frac{1}{\pi} \int_0^\infty d\beta \frac{1}{\pi} \int_0^\infty d\eta \frac{1}{\pi} \int_0^\infty d\mu \int \int \int dx'dy'dz' c(x',y',z',0) e^{-(\mu^2+\eta^2+\beta^2)dt} \times \]

\[
\cos(\beta(x-x')) \cos(\eta(y-y')) \cos(\mu(z-z')). \quad (Eq. 2.32)
\]

Given that

\[
\int_0^\infty e^{-s^2} \cos bs \, ds = \frac{\sqrt{\pi}}{2} e^{-b^2} \quad (Eq. 2.33)
\]

and applying variables substitutions such as \( s = \beta \sqrt{Dt} \), \( ds = \sqrt{Dt} \, d\beta \), \( b = \frac{x-x'}{2Dt} \), and \( 2bs = (x-x')\beta \), integrating over \( \beta, \eta, \) and \( \mu \) with the initial conditions yields

\[
c_{3D}(x,y,z,t) \equiv c(x,y,z,t) = \frac{1}{\sqrt{4Dt}} \int_{-a}^{a} dx' e^{-\frac{(x-x')^2}{4Dt}} \frac{1}{\sqrt{4Dt}} \int_{-a}^{a} dy' e^{-\frac{(y-y')^2}{4Dt}} \frac{1}{\sqrt{4Dt}} \int_{-a}^{a} dz' e^{-\frac{(z-z')^2}{4Dt}}. \quad (Eq. 2.34)
\]

Solving the Gaussian integrals in terms of error functions where

\[
\text{erf}(h) = \frac{2}{\sqrt{\pi}} \int_0^h e^{-p^2} \, dp \quad (Eq. 2.35)
\]

and applying variable substitutions such as \( p = \frac{x-x'}{\sqrt{4Dt}} \) and \( dp = \frac{-dx'}{\sqrt{4Dt}} \), the concentration profile in three dimensions becomes,

\[
c_{3D}(x,y,z,t) = \frac{1}{2} \left[ \text{erf}(\frac{x+a}{\sqrt{4Dt}}) - \text{erf}(\frac{x-a}{\sqrt{4Dt}}) \right] \frac{1}{2} \left[ \text{erf}(\frac{y+a}{\sqrt{4Dt}}) - \text{erf}(\frac{y-a}{\sqrt{4Dt}}) \right] \frac{1}{2} \left[ \text{erf}(\frac{z+a}{\sqrt{4Dt}}) - \text{erf}(\frac{z-a}{\sqrt{4Dt}}) \right]. \quad (Eq. 2.36)
\]

The FRAP recovery then can be calculated by integrating over the concentration profile, yielding the recovery in its most general form where \( d=1,2,3 \) denotes the
dimension.

\[ FRAP_d(t) = 1 - \left[ \text{erf} \left( \frac{a}{\sqrt{Dt}} \right) - \frac{1}{\sqrt{\pi}} \left( 1 - e^{-a^2/2Dt} \right)^{\frac{1}{2}} \right]^d \]  

(Eq. 2.37)
3 Analysis and Modeling of Vesicle Dynamics Show that Actin-Based Transport Is Essential for Tip Growth

3.1 Abstract
Filamentous actin has been shown to be essential for tip growth in an array of plant models, including *Physcomitrella patens*. One hypothesis is that diffusion can transport secretory vesicles, while actin plays a regulatory role during secretion. Alternatively, it is possible that actin-based transport is necessary to overcome vesicle transport limitations to sustain secretion. Therefore, a quantitative analysis of diffusion, secretion kinetics and geometry is necessary to clarify the role of actin in polarized growth. Using FRAP analysis, we first show that secretory vesicles move toward and accumulate at the tip in an actin-dependent manner. We then depolymerized F-actin to decouple vesicle diffusion from actin-mediated transport, and measured the diffusion coefficient and concentration of vesicles. Using these values, we constructed a theoretical diffusion-based model for growth, demonstrating that with fast-enough vesicle fusion kinetics, diffusion could support normal cell growth rates. We further refined our model to explore how experimentally-extrapolated vesicle fusion kinetics and the size of the secretion zone limit diffusion-based growth. This model predicts that diffusion-mediated growth is dependent on the size of the region of exocytosis at the tip, and that diffusion-based growth would be significantly slower than normal cell growth. To further explore the size of the secretion zone, we used a cell wall-degradation enzyme cocktail, and determined that the secretion zone is smaller than 6 μm in diameter at the tip. Taken together our results highlight the requirement for active
transport in polarized growth and provide important insight into vesicle secretion during tip growth.

3.2 Introduction
Precise regulation of exocytosis is essential to maintain polarized cell growth in a variety of plant systems. Cell polarized growth, or tip growth, is a process by which a cell grows in a unidirectional manner and is found ubiquitously throughout the plant kingdom (Hepler et al., 2001). Pollen tubes, root hairs, and moss protonemal cells have all emerged as models for this process (Hepler et al., 2001; Menand et al., 2007). To achieve polar expansion in the presence of uniform turgor pressure, these cells must spatially regulate the extensibility of the cell wall (Winship et al., 2010; Hepler et al., 2013). This is achieved through the polarized exocytosis of various cell wall materials and loosening enzymes (Rojas et al., 2011).

How plant cells establish spatially directed exocytosis during polarized growth has been the focus of a number of studies in the past decade (Cardenas et al., 2008; McKenna et al., 2009; Moscatelli et al., 2012). Many of these efforts have heavily implicated the cytoskeleton as a key player in exocytosis. Evidence suggests that myosin XI transports vesicle containing cell wall materials via filamentous actin (F-actin) to the growing tip of the cell (Vidali et al., 2010; Madison and Nebenfuhr, 2013; Madison et al., 2015). In pollen tubes, a cortical actin fringe several microns behind the cell tip has been shown to be essential in polarized growth (Vidali et al., 2001; Lovy-Wheeler et al., 2005; Vidali et al., 2009). Rounds et al. have shown that the presence of this fringe is necessary for the focusing of apical pectin deposition (Rounds et al., 2014). However, work with FM dyes
supports the hypothesis that exocytosis happens along an annulus behind the cell tip (Bove et al., 2008; Zonia and Munnik, 2008). In moss protonemal cells, the actin cytoskeleton concentrates at the extreme cell apex (Vidali et al., 2009). At this extreme apex, vesicle fluctuations have been shown to predict F-actin fluctuations (Furt et al., 2013). Furthermore Myosin XI, which is essential for tip growth (Vidali et al., 2010), can also anticipate actin fluctuations (Furt et al., 2013). ROP GTPases, which have been thought to initiate tip growth (Lee et al., 2008), have been shown to influence apical filamentous actin dynamics and concentrations (Burkart et al., 2015).

Significant work has been done to probe the molecular players involved in cytoskeletal mediated exocytosis, however, there is a growing need to demonstrate a mechanistic link between F-actin and polarized growth (Rounds and Bezanilla, 2013). Although the actin fringe has been modeled in pollen tubes (Sanati Nezhad et al., 2014), to the best of our knowledge, the role of apical F-actin in other, slower polar growth systems, is yet to be examined. What are the physical limitations an active transport system like the actin cytoskeleton must overcome to facilitate vesicle exocytosis and sustain polarized growth? Vesicle concentrations and diffusion coefficients, exocytic reaction kinetics, cell growth rates, and the size of the active region of exocytosis, all place specific limitations on the actin-based transport system. Quantifying these fundamental requirements will provide key insight into understanding the requirement for transport in this system.
Without a quantitative assessment of the potential physical limitations outlined above, we can hypothesize several functions for the actin cytoskeleton. For example, it could serve as a means to overcome slow vesicle diffusion limitations to drive exocytosis. The actin cytoskeleton could also function to surpass slow reaction kinetics associated with vesicle fusion events on the plasma membrane. It is also possible that exocytosis is confined to a relatively small area on the plasma membrane; the actin cytoskeleton could then function as a means to focus vesicles to this small exotic zone. Finally, it also remains a possibility that F-actin active transport is not required to sustain polarized growth.

To better understand how F-actin influences vesicle transport, we fluorescently labeled the v-SNARE, VAMP72 (Sanderfoot, 2007) in the moss *Physcomitrella patens* (Vidali and Bezanilla, 2012). *P. patens* was chosen here because it does not exhibit large organelle cytoplasmic streaming (Shimmen, 2007) which can complicate the analysis of vesicle transport (Furt et al., 2012). Instead, *P. patens* only exhibits two modes of vesicle transport, namely diffusion and active transport along the cytoskeleton. We visualized these modes of transport, and performed Fluorescence Recovery After Photobleaching (McNally, 2008; Loren et al., 2015), FRAP, during polarized growth. To probe the utility of actin-mediated active transport we used the small molecule inhibitor latrunculin B to depolymerize actin and uncouple vesicle diffusion from active transport. With FRAP, and Number and Brightness analysis (Digman et al., 2008; Loren et al., 2015)Kingsley, Bibeau et al., see section 3), we measured vesicle diffusion rates and concentrations, respectively. We then developed a diffusion-limited analytical
model, and numerically solved it using these parameters, as well as previously measured cellular growth rates (Furt et al., 2013). Using this model, we quantitatively explored the limiting factors associated with cell growth, and used enzymatic digestions of the cell wall to further constrain the model.

3.3 Results

3.3.1 Depolymerization of Actin Stops Cell Growth in Minutes

Previous evidence in plants suggests that actin filaments are essential for polarized growth (Vidali et al., 2001). It remains unclear, however, if removal of the actin cytoskeleton immediately abolishes growth, or if some growth, supported by diffusion, continues to happen once actin is removed. To address this question, we treated growing protonemal cells with the actin monomer sequestering agent latrunculin B to depolymerize the actin filaments (Vidali et al., 2001; Vidali et al., 2009). To visualize vesicle accumulation, we used a cell line expressing 3mEGFP-VAMP72 (Furt et al., 2013). Following latrunculin B treatment, we could not detect any tip growth (Figure 3.1A), which is consistent with previous work (Vidali et al., 2001; Vidali et al., 2009). Treatment also abolished tip localization of VAMP72-vesicles (Figure 3.1B) and produced an intensity gradient that decreased toward the cell tip (Figure 3.1C). Vehicle-treated controls grew at 5.5 ± 0.4 nm/s, which is within the range of previously measured growth rates (Furt et al., 2013). Since latrunculin B arrests growth, we concluded that cell growth could not continue
without filamentous actin.

Figure 3.1. Latrunculin B stops growth. A) Representative kymographic analysis of caulonemal cells expressing 3mEGFP-VAMP72 vesicles before (top) and after (bottom) vehicle (left) or 5 μM latrunculin B treatment (right). Black vertical arrow indicates vertical time axis. Horizontal arrow denotes treatment time. White space between top and bottom figures represents the approximate time necessary to apply the treatment to the culture. Dotted white box indicates region for line scan. B) Representative cells before (top) and after (bottom) latrunculin B treatment. Scale bar is 5 μm. C) Measured intensity gradient for latrunculin B treated cells. Measurement was taken from the white dotted box in (B) for each measured cell. (error bars indicate standard error, and n = 4)
3.3.2 Vesicles Exhibit Active Transport at the Cell Tip

Since the depolymerization of filamentous actin immediately blocks growth, we sought to determine if actin plays a role in vesicle transport at the cell tip. To this aim, we performed fluorescence recovery after photobleaching experiments on a cell line expressing the vesicle marker 3mCherry-VAMP72 (Furt et al., 2013) at the cell tip (Figure 3.2A), and a region distal to the tip, subsequently referred to as the shank. We chose this cell line because we found that 3mCherry is more sensitive to photobleaching than 3mEGFP. To better understand the differences in molecular flux at the tip and the shank, fluorescence recoveries were corrected for acquisition photobleaching (Figure 3.3) and normalized to the mean prebleach shank intensity. Since VAMP72-vesicles localize to the cell tip (Figure 3.2B, and Figure 3.4)—likely through myosin binding to actin—we expected a reduction in the recovery after photobleaching of vesicles at the cell tip. Instead VAMP72-vesicles exhibited a faster exchange of particles at the tip than the shank (Figure 3.2A), demonstrating that the accumulation of vesicles at the cell tip is not simply due to static capture. This indicates that there is a stream of active transport at the cell tip that is faster than the recovery at the shank.
To better understand the directionality of this fast active transport, we recorded the movement of VAMP72-vesicles after photobleaching. After averaging several fluorescent recoveries we found that these vesicles moved along the cell cortex toward the tip of the cell (direction of recovery denoted by white arrows in Figure 3.2B). We further quantified this directional recovery by measuring the fluorescence intensity along the perimeter of the photobleaching region of interest (Figure 3.2B). At early times ($\Delta t_1 = 0.7 - 1.6 \text{s}$) after photobleaching we found

![Figure 3.2](image)

**Figure 3.2.** VAMP72-vesicle dynamics during polarized growth. A) Fluorescence recovery of 3mCherry-VAMP72-vesicles at the cell shank (green circles) and cell tip (black squares). $n = 8$ and 10, respectively (error bars indicate standard deviation). B) Cropped and frame averaged photobleaching ROI at the cell tip for 3mCherry-VAMP72-vesicles (top) and simulation (bottom) $n = 8$ and 50, respectively. Image intensity denoted with rainbow lookup table; $\Delta t_1 = 0.7 - 1.6 \text{s}$ and $\Delta t_2 = 20 - 40 \text{s}$. White arrows mark direction of recovery (left). White circle and arrow denotes how the perimeter of the ROI was measured (right). C-D) Intensity profiles of 3mcherry-VAMP72 along the ROI perimeter at the cell tip during the time intervals $\Delta t_1 = 0.7 - 1.6 \text{s}$ (C) and $\Delta t_2 = 20 - 40 \text{s}$ (D) ($n = 10$, error bars indicate standard error).
that the maximum fluorescence intensity was cortical but moving toward the cell tip (Figure 3.2B and 3.2C). Finally, at longer time intervals \((\Delta t_2 = 20 - 40 \text{s})\) VAMP72-vesicles became localized to the cell tip (Figure 3.2B and 3.2D). Since these vesicles exhibited an increased flux at the cell tip when compared to the shank, and because the vesicles recovered along the cell cortex toward the tip, we conclude that active transport drives a significant portion of the movement of VAMP72 labeled vesicles.

Figure 3.3. Image acquisition photobleaching/reversible photoswitching and correction. A) Image acquisition photobleaching for latrunculin B treated cells expressing 3mCherry-VAMP72-vesicles at the cell tip (left) and shank (right). Red squares represent mean acquisition photobleaching intensity. Time variant sampling rate indicated above. Blue dashed lines represents best fit piecewise exponential of acquisition photobleaching. Error bars represent standard error. \((n=9)\). B) Fluorescence recovery before (black triangles) and after (red squares) acquisition photobleaching correction for latrunculin B treated cells expressing 3mCherry-VAMP72-vesicles at the cell tip (left) and shank (right). \(n=8\) and 6, respectively.
3.3.3 Vesicles Undergo Diffusion when Uncoupled from Actin

To determine if active vesicle transport is actin dependent, and to quantify vesicle diffusion, we uncoupled vesicle diffusion from actin-mediated transport with the actin depolymerizing agent latrunculin. This treatment abolishes the tip localization of VAMP72-vesicles (Figure 3.1 and Figure 3.4), suggesting that untreated VAMP72 labeled vesicles exhibit active transport in an actin dependent manner. To accurately quantify vesicle diffusion, we performed photobleaching at the cell tip and shank, and (as done in sections 2.5.6 and 2.5.7) performed corrections on the recovery curves to remove the effects of acquisition photobleaching and reversible photoswitching (Figure 3.3). Fluorescence recovery curves for the two specific locations were normalized to their respective prebleach intensities. Following photobleaching, the cell tip exhibited a reduced fluorescence recovery rate when compared to the cell shank (Figure 3.5A). Because photobleaching at the cell tip was close to the cell membrane, we expected the diffusion based recovery at the tip to be limited by this boundary, and as a consequence slower.

![Figure 3.4. Latrunculin B abolishes tip localization of VAMP-vesicles. A) Representative cells expressing 3mCherry-VAMP72 vesicles before (top) and after latrunculin B (bottom). B) 3mCherry-VAMP72 fluorescence line scans through untreated (black) and latrunculin B (red) cells. Dashed lines indicate the position of bleaching ROI. (error bars indicate standard error n>=6)](Image)
This is not the case at the cell shank where fluorescence recovery can happen from all directions. To accurately determine the diffusion coefficient, we used a particle-based computational model of FRAP that incorporates the properties of our confocal imaging system, the three-dimensional moss cell shape, and the thermal fluctuations of the fluorophore (see section 3).

To reduce the number of free parameters in this FRAP model, we

---

**Figure 3.5.** Latrunculin B treated VAMP72-vesicle dynamics. A) Fluorescence recovery of latrunculin B treated 3mCherry-VAMP72-vesicles at the cell shank (green circles) and cell tip (black squares) with corresponding best fit simulation results (red). \( n = 6 \) and 8, respectively (error bars indicate standard deviation). B) Cropped and frame averaged photobleaching ROI at the cell tip for latrunculin B treated 3mCherry-VAMP72-vesicles (top) and simulation (bottom) \( n = 8 \) and 50, respectively. Image intensity denoted with rainbow lookup table; \( \Delta t_1 = 0.8 - 2.5 \) s and \( \Delta t_2 = 20 - 40 \) s. White arrows mark direction of recovery (left). White circle and arrow denote how the perimeter of the ROI was measured (right). C-D) Intensity profiles of latrunculin B treated VAMP72 along the ROI perimeter at the cell tip during the time intervals \( \Delta t_1 = 0.8 - 2.5 \) s (C) and \( \Delta t_2 = 20 - 40 \) s (D) with corresponding simulations in red (\( n = 8 \), error bars indicate standard error).
experimentally measured the point spread function of our confocal system, and the bleaching width of the confocal laser (Figure 3.6). The FRAP model recovery curves were also corrected for reversible photobleaching to better match experimental results (see Methods). The two remaining open parameters in our

![Figure 3.6. Point Spread Function (PSF) for imaging and photobleaching. A) Confocal PSF for red beads and the corresponding Gaussian approximation of the beam. B) Photobleached 3mCherry-VAMP72-vesicles treated with formaldehyde. Photobleaching region is shown in solid white. Horizontal line scans were acquired within the dashed yellow rectangle and averaged in the vertical direction. (top) Single parameter fit to experimental formaldehyde bleaching profile with imaging \(w_0\) and best fit bleaching proportionality coefficient \(K\). (middle) Two parameter photobleach model fit to experimental formaldehyde bleaching profile with best fit parameters \(w_0\) and bleaching proportionality coefficient \(K\). Error bars indicate standard error. (n=8).]
model were the diffusion coefficient and the photobleaching proportionality constant that relates laser intensity to the number of fluorophores bleached. After generating simulations for an array of these two parameters, we employed a fitting routine to determine the best-fit parameters (see Materials and Methods). This analysis showed that a single diffusion coefficient ($D = 0.29 \pm 0.14 \mu m^2 s^{-1}$) was sufficient to account for the fluorescence recovery seen both at the tip and the shank.

To further demonstrate that our best-fit particle-based simulation results accurately represents the motion of VAPM72-vesicles in vivo, we compared the directional fluorescence recovery of the best-fit particle-based simulation (without the reversibly photobleached fraction) to the experimental fluorescence recovery. At early times of fluorescence recovery, an intensity gradient was present within the photobleached regions for both the experiments and simulations (Figure 3.5B) following limited volume corrections (Figure 3.7). The gradient decayed toward the cell tip (Figure 3.5C) and flattened at long times (Figure 3.5D), which suggests that the cell boundary restricts the spatial recovery of VAMP72-vesicles. This gradient was also observed in our simulations of FRAP at the cell tip (Figure 3.5E and 3.5F), but not at the cell shank (Figure 3.8). This indicates that vesicle recovery at the cell tip is influenced by its position next to the apical plasma membrane, such that material cannot flow in or exchange in all directions. Importantly, this recovery
closely matches a diffusion model (Figure 3.5E and 3.5F), further supporting our claim that VAMP72-vesicles exhibit diffusion.

### 3.3.4 Analytical Modeling Demonstrates Cylindrical Cell Growth Is not Diffusion Limited

To determine if diffusion alone can supply enough vesicles to the growing cell edge to sustain polarized growth in *P. patens*, we initially created a simple analytical model of diffusion-based cell growth in the absence of the actin cytoskeleton. To build this model, we first determined the number of vesicle fusion events per second, $\phi$, needed to support normal cell growth (Bove et al., 2008). We assumed that *P. patens* is cylindrical in shape at the shank with an outer cell radius of $R_0 \sim 6 \mu m$, and a wall thickness $d \sim 250 \text{ nm}$ (Martin et al., 2009). As the cell grows, the wall maintains its outer radius $R_0$ and thickness $d$, and elongates at a rate of \( \dot{L}_y = 5.5 \pm 0.4 \text{ nm/s} \). The wall volume per second, $V_{w'}$, needed to sustain this growth can be written as,
Assuming that the cell wall materials within a vesicle do not change in volume once they are incorporated into the wall, the number of vesicle fusion events needed to support growth can be written as,

$$\phi = \frac{\dot{V}_w}{V_v}.$$  \hspace{1cm} (Eq. 3.2)

Here $\phi$ is the number of vesicle fusion events as introduced above, and $V_v$ is the 

$$\dot{V}_w \equiv \frac{dV_w}{dt} = \dot{I}_g \pi [R_0^2 - (R_0 - d)^2].$$ \hspace{1cm} (Eq. 3.1)
volume of a vesicle. This assumption is supported by electron micrographs in pollen tubes that show that the electron densities of vesicles and the cell wall are similar (Lancelle and Hepler, 1992; Derksen et al., 1995). Assuming that secretory vesicles are spherical in shape with a radius of a 40 nm (Lancelle and Hepler, 1992), one can calculate that \( v_v \sim 2.68 \times 10^{-4} \mu m^3 \) and \( \dot{V}_w \sim 0.051 \mu m^3 s^{-1} \), and therefore, the vesicle fusion rate for a growing cell is \( \phi \sim 189 \text{ vesicles/s} \).

To relate observed vesicle diffusion to this calculated vesicle fusion rate, we modeled the growing cell as a cylinder with an absorbable boundary at one face of the cylinder. This model is equivalent in principle to latrunculin B treated cells, in which diffusion alone must support growth. Here the cylinder is oriented so that the tip is positioned at \( x = 0 \) and the back end of the cylinder is at \( x = L \) (Figure 3.9A and 3.9B). The cylinder contains a population of vesicles with a spatial concentration that can be written as \( c(x, t) \). In wild type \( P. patens \) polarized growth, we determined that large organelles such as chloroplasts and the vacuole remain at a distance \( L = 16 \pm 1.4 \mu m \) behind the growing tip. The presence of these large organelles allows us to make two important simplifications. First, the constant distance from these organelles to the tip makes it reasonable to model the growing tip of the cell as a cylinder of constant size. Second, we assume these organelles act as a reflective boundary (at \( x = L \)) and block the flux of vesicles through this edge, \( c'(L, t) = 0 \).

At the opposite edge of the cylinder (at \( x = 0 \)), we assume perfect vesicle absorbability (e.g. infinite on-rate), simulating a possible maximum limit for growth rate. This assumption of infinite on-rate (or presumably unlimited supply of
receptors) uncouples vesicle diffusion limitations from receptor-mediated vesicle-membrane fusion limitations by assuming that vesicles instantly fuse upon contact with this face of the cylinder. This is represented by the vesicle concentration at the extreme edge being zero, i.e. \( c(0,t) = 0 \). To model vesicle production, we assume a spatially homogenous production rate \( Q \). We impose this uniform production rate because of the almost uniform distribution of Golgi bodies in the cell (Furt et al., 2012). We can then express the concentration of vesicles \( c(x,t) \) as a time-dependent diffusion equation with a constant production rate \( Q \), i.e.,

\[
\frac{\partial c(x,t)}{\partial t} = \nabla \cdot [D(x)\nabla c(x,t)] + Q. \tag{Eq. 3.3}
\]

Here \( \nabla c(x,t) \) represents the vesicle concentration gradient. At steady state growth, we can take \( \frac{\partial c(x,t)}{\partial t} = 0 \). Assuming homogeneous diffusion \( (D = \text{constant}) \), the radial symmetry of the cell lets us define the concentration throughout the cell as dependent only on the linear position \( x \). This produces a general solution for \( c(x) \) of the form

\[
c(x) = -\left( \frac{Q}{2D} \right)x^2 + K_1x + K_2, \tag{Eq. 3.4}
\]

where \( K_1 \) and \( K_2 \) are constants determined by the boundary conditions. Solving for the two boundary conditions ( \( c(0) = 0 \) and \( c'(L) = 0 \) ) produces a profile dependent on the vesicle production rate \( Q \) such that

\[
c(x) = -\left( \frac{Q}{2D} \right)x^2 + \left( \frac{QL}{D} \right)x. \tag{Eq. 3.5}
\]

There is an additional constraint that fixes the value of \( Q \)—this steady state
condition requires that the concentration of vesicles at the cell shank $c(L)$ is equal to a predetermined concentration $c_L$. This constraint produces an expression for $Q$, namely,

$$Q = 2Dc_L/L^2. \quad (\text{Eq. 3.6})$$

During steady state growth, the vesicle production rate $Q$ must match the number of vesicle fusion events $\phi$ such that,

$$\phi = QL\pi R^2. \quad (\text{Eq. 3.7})$$

Here, $R$ is the radius of the growing cylinder. This expression allows us to determine the number of vesicle fusion events for any given $c_L$, $D$, $R$ and $L$, and the values used for these four parameters are listed in Table I. Diffusion coefficient, $D$, was measured as described in section 2 with FRAP analysis. To determine $c_L$, we used our confocal particle-based simulation (see section 2) to produce a range of potential concentrations, and found that resolving individual particles becomes impossible at concentrations above $10 \text{vesicles/}\mu\text{m}^3$ (Figure 3.9C). Since we cannot resolve individual particles experimentally, we let this be the lower bound estimate for $c_L$. Finally, we can relate the vesicle fusion rate predicted by our analytical model in Eq. (3.7) to the observed vesicle fusion rate calculated using Eq. (3.2).
Table 4.1 Analytical Model Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion Coefficient, $D$</td>
<td>$0.29 \pm 0.14 \mu m^2 s^{-1}$</td>
<td>Measured with FRAP</td>
</tr>
<tr>
<td>Cylinder length, $L$</td>
<td>$16 \pm 1.4 \mu m$</td>
<td>Measured from microscope images (n=8)</td>
</tr>
<tr>
<td>Cylinder Radius, $R$</td>
<td>$6 \pm 0.2 \mu m$</td>
<td>Measured from microscope images (n=8)</td>
</tr>
<tr>
<td>Concentration at $c(L)$, $c_L$</td>
<td>$10 - 100 \text{ves}/\mu m^3$</td>
<td>Estimated from Figure 3.1B</td>
</tr>
</tbody>
</table>

For the range of possible values of $c_L$, the model predicts two consistent effects that influence cell growth. First, there is a parabolic reduction in vesicle concentration from the shank out toward the growing tip (Figure 3.9D). Additionally, we find a linear relationship between cell growth rates and $c_L$. At the lower bound vesicle concentration $c_L = 10 \text{vesicles}/\mu m^3$, the predicted vesicle fusion rate is $\phi = 41 \pm 20 \text{vesicles}/s$ and the corresponding cell growth rate is $1.2 \pm 0.6 \text{nm/s}$ (Figure 3.9E and 3.9F). At the upper bound vesicle concentration $c_L = 100 \text{vesicles}/\mu m^3$, $\phi = 410 \pm 201 \text{vesicles}/s$ and the growth rate is $12 \pm 6 \text{nm/s}$. The confidence bounds for these estimates are the result of lower and upper bound estimates for the vesicle diffusion coefficient (see Materials and Methods). This growth rate indicates that diffusion cannot sustain cell growth at our estimated lower bound vesicle concentrations (Figure 3.9E) but, can easily support growth at our upper bound estimate (Figure 3.9F).
This means that diffusion limitations at the growing cell edge may not serve as the only reason why these cells stop growing. Interestingly this implies that under idealized vesicle fusion and vesicle concentrations, diffusion could support cell growth. Additional factors such as membrane-vesicle fusion kinetics, small

Figure 3.9. Differential equation model of diffusive cell growth with idealized geometry. A) Diagram of the cylindrical approximation of the moss geometry. Green circles represent vesicles. Red boundaries are reflective boundaries and green boundary is perfectly absorbable. B) Representative bright field image of growing cell with cylindrical approximation of analytical model in red. Scale bar is 5 μm. C) Simulated 3mEGFP-VAMP72-vesicles with $c_L = 10 \text{ vesicles/μm}^3$. Scale bar is 4 μm. D) Normalized concentration profile for the solution of the analytical model, Eqs. (3)-(7). E+F) Growth rates for the analytical model, Eqs. (3.3)-(3.7), solved for $c_L = 10 \text{ vesicles/μm}^3$ (E) and $c_L = 100 \text{ vesicles/μm}^3$ (F). Model error bars represent predicted growth rates within 95% confidence intervals, with $D = 0.15 \text{ μm}^2 s^{-1}$ and $D = 0.43 \text{ μm}^2 s^{-1}$, respectively. Wild type error bars represent 95% confidence intervals, $n = 4$. 

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active exocytic regions, and the three-dimensional cell geometry may also prevent latrunculin B treated cells from growing.

3.3.5 Vesicle Concentrations Yield an Estimate of Vesicle Fusion Kinetics
To explore the additional factors that may limit cell growth and better determine the concentration of VAMP72 labeled vesicles during latrunculin B treatment, we used Numbers and Brightness (N&B) analysis (Digman et al., 2008). In N&B analysis, the mean and variance of a fluorophore’s intensity fluctuations are used to determine the concentration of molecules in solution. The foundation of this analysis is the assumption that molecule number fluctuations in a given volume are Poisson distributed—which holds true for a freely diffusing population of vesicles (Brown et al., 2008). Since the mean and variance of a Poisson distribution are equal, it is possible to algebraically solve for the brightness of a fluorophore even in the presence of detector shot noise (Digman et al., 2008). Using this analysis, we found that the vesicle concentrations of latrunculin B treated cells were $39 \pm 6$ and $38 \pm 2$ vesicles/μm$^3$ at the tip and shank, respectively (Figure 3.10, see Methods for more details). No statistically significant difference in concentrations were found at the tip and shank ($p-value = 0.8349, n = 6$). At these vesicle concentrations the analytical model predicts growth at $5 \pm 2.9$ nm/s, similar to normal growth.

Since diffusion-based growth with ideal vesicle fusion kinetics is enough to support cell growth, we sought to estimate the true kinetics of vesicle membrane fusion during exocytosis. Docking and fusion of exocytic vesicles is a multistep process mediated by protein complexes such as the Exocyst (Kulich et al., 2010;
Bloch et al., 2016) and the SNARE complex (Lipka et al., 2007; El Kasmi et al., 2013); which we simplify by assuming VAMP72-vesicles interact with one type of receptor on the plasma membrane at the cell tip. We also assume that this type of receptor has one reaction rate, and facilitates the integration of vesicles into the plasma membrane. This allows us to write the flux equation through the plasma membrane as follows (Phillips, 2013),

\[ \phi = mK_{on}c(0) \quad \text{(Eq. 3.8)} \]

Here \( m \) is the total number of receptors on the plasma membrane at the cell tip, \( K_{on} \) is the binding reaction rate between vesicles and the receptors, and \( \phi \) is the measured number of vesicle fusion events per second (as previously defined with Eq. (3.2)). As a first approximation, and for the most parsimonious case, we assume that \( mK_{on} \) is constant during growth. Since the intensity of vesicles at the very cell tip, \( c(0) \), for growing cells is roughly two-fold higher than \( c(0) \) for latrunculin B treated cells (Figure 3), we can solve Eq. (3.8) to get \( mK_{on} \sim 2.5 \, \text{s}^{-1} \, \mu m^3 \).
3.3.6 Refined Diffusion Model Illustrates the Requirement for F-actin in Polarized Growth

With relevant vesicle concentrations and fusion kinetics known, we then built a more comprehensive model to determine the physiological conditions under which diffusion could or could not sustain cell growth, and developed an insight into the limiting factors in polarized growth from a vesicle trafficking perspective. To this end, we created a diffusion-based growth model without the actin cytoskeleton. To incorporate a more realistic geometry of the growing moss cell tip, we used the

![Figure 3.10](image)

**Figure 3.10.** Brightness and numbers analysis reveals vesicle concentrations. A) Representative 6X6 μm image stack of latrunculin B treated 3mEGFP-VAMP72 vesicles at the cell shank. B) Fluorescence fluctuations of 3mEGPF-VAMP72 vesicles at a single pixel from (A). C) Representative 2-dimensional scatter plot of pixel brightness and intensity from (A). Blue to yellow lookup table was used to denote frequency. D) Volume of point spread function calibration. Black circles indicate simulated data. Red dotted line indicates standard curve. Blue diamond indicates representative experimental concentration from (A).
finite element analysis modeling software Comsol Multiphysics (Comsol Inc, Stockholm, Sweden) to solve Eq. (3.3) within the moss geometry for biologically relevant boundary conditions. We used impermeable (reflective) boundary conditions for most of the plasma membrane, except for the active region of exocytosis at the cell tip, where the \( m \) receptors were concentrated. In this region we used Eq. (3.8) as a boundary condition to simulate diffusion-mediated exocytosis. Since the size of the zone of vesicle exocytosis is unknown, we simulated four different discrete exocytic zones, centered at the cell apex, with diameters \( \Omega = 1, 2, 4, \) and \( 10 \) \( \mu \)m (Figure 3.11A). To satisfy Eq. (3.8), each of the four simulated exocytic zones maintained the same total number of receptors, \( m \), but at different densities. Although it has been previously shown that vesicle exocytic zones may have a monotonically increasing \( mK_{on} \) focused at the tip (Campàs and Mahadevan, 2009), we simulate \( \Omega \) with a constant \( mK_{on} \) to establish the effective length scales for the exocytic zone.

To achieve growth rates similar to a wild type cell, our model predicts that a cell growing by diffusion would have to maintain vesicle concentrations at the shank much greater than those observed experimentally. Specifically, these concentrations range between \( \sim 125 \) and \( \sim 477 \) vesicles/\( \mu \)m\(^3\) at the cell shank, \( c_L \), while our experimentally measured shank concentration is \( 38 \pm 2 \) vesicles/\( \mu \)m\(^3\). Based on the size of the exocytosis zone we found that these concentrations exhibited different gradients as they approached the cell tip. Large exocytic zones produced concentration gradients that fell slowly as they approached the cell tip, while smaller zones produced concentration gradients that rapidly fell as they
approached the cell tip (Figure 3.11B and 3.11C). We also found that larger exocytic zones could support cell growth at lower vesicle concentrations than smaller exocytic zones. This means that a cell with larger exocytic zones could grow more quickly than a cell with a smaller exocytic zone, if both cells had the same steady state vesicle concentrations. This is because larger exocytic zones have a larger vesicle capture area and are less diffusion limited. For large exocytic regions and experimentally measured vesicle concentrations, diffusion alone could lead to cell growth rates slightly slower than $2 \text{ nm/s}$ (Figure 3.11E). Smaller exocytic regions at experimentally relevant vesicle concentrations produced growth rates slower than $1 \text{ nm/s}$ (Figure 3.11E). Although actin could serve other functions associated with growth, our results demonstrate that there is a specific requirement for active transport to sustain cell growth, and that this need is dependent of the size of the exocytic zone.
Figure 3.11. Differential equation model of diffusive growth with moss cell geometry. A) Plasma membrane with the active area of exocytosis marked in red. Black line indicates reflective boundary. Dashed line indicates the exocytosis region diameter $\Omega$. B) Model predicted steady-state vesicle concentration profile necessary to sustain wild type cell growth. Color table indicates concentration gradients with high concentrations as warm colors and low concentrations as cool colors. C+D) Normalized (C) and unnormalized (D) concentration profiles from (B) necessary to sustain wild type cell growth. (B). E) Comsol model predicted growth rates for experimentally measured shank concentrations for $\Omega = 10, 4, 2, \text{ and } 1 \mu m$ from left to right. Model error bars represent predicted growth rates for $D = 0.15$ and $D = 0.43 \mu m^2 s^{-1}$, respectively. Wild type error bars represent standard error of the mean, $n = 4$. 
3.3.7 Wall Extensibility is Weakest at the Cell Tip

In order to constrain our diffusion model with an experimentally relevant secretion size, we examined the material properties of the cell wall. To probe these properties during polarized growth, we used the enzyme cocktail driselase, which enzymatically degrades the wall. When exposed to driselase, we found that the wall of the tip-growing cells would begin to extrude and eventually fail (Figure 3.12A). Following treatment extrusion and failure was observed within minutes. We found that the average size of the extruded region was approximately $5.8 \pm 0.5 \, \mu m$ in diameter (Figure 3.12B), and its center was within $3 \, \mu m$ from the center of the cell (Figure 3.12C). Assuming degradation happens uniformly, this indicates that the cell wall is most extensible at the cell tip. Due to the fact that exocytosis mediates wall extensibility, we inferred that exocytosis likely takes place within a region smaller than $5.8 \, \mu m$ in diameter. Applying this upper limit estimate of the secretion zone to the diffusion-based cell growth model, the model predicts that the cell could never grow faster than $2 \, nm/s$ (Figure 3.12D and 3.12E), which is significantly less than $5.5 \pm 0.4 \, nm/s$ observed experimentally.
Figure 3.12. Driselase reveals cell wall extensibility. A) Representative time series of cell wall rupture following exposure to driselase. Cell perimeter is highlighted in blue. Green circles mark the end points of the rupture arc length. Black circles mark the cell tip. Red circle shows maximally deflected rupture potion. Red star shows the projected rupture position. Scale bar is 5\( \mu m \). Dashed line indicates the rupture diameter \( \Omega \). B) Cumulative frequency of rupture diameter \( \Omega \). Insert displays a box plot of the rupture diameter. Median is marked in red, the quartiles are marked in blue, and the minimum and maximums are in black. Red dot is the mean. C) Location of cell rupture along the edge. Blue dots indicate the cell boundary. Red stars indicate region of rupture. \( n = 17 \). D) Comsol model predicted concentration profile, necessary to sustain wild type cell growth, for \( \Omega = 5.8 \mu m \). E) Comsol model predicted growth rate for experimentally measured shank concentrations and \( \Omega = 5.8 \mu m \), compared to wild type growth rate. Model error bars represent predicted growth rates for \( D = 0.15 \) and \( D = 0.43 \mu m^2 s^{-1} \), respectively. Wild type error bars represent standard error of the mean, \( n = 4 \).
3.4 Discussion

This work demonstrates that in moss protonemata actin drives vesicle transport, and shows quantitatively that this transport is necessary to sustain normal cell growth. Specifically, the experimentally measured vesicle diffusion coefficient, vesicle concentrations, membrane reaction kinetics, and size of the exocytosis region collectively impose enough limitations on diffusion based growth to require an active transport system in polarized secretion, Figure 3.13.

While it is well-known that filamentous actin is necessary for polarized growth, here we showed that latrunculin B treatments halt tip growth within 2 minutes, indicating an immediate reliance on actin. We then showed with FRAP that VAMP72 labeled vesicles are transported cortically to the cell apex in an actin-dependent manner. Nevertheless, this FRAP analysis is limited in that it does not have the spatiotemporal resolution to infer additional modes of vesicle dynamics, such as transport to the plasma membrane and/or endocytic vesicle resupply.

Depolymerizing the actin cytoskeleton with latrunculin B, reduced the number of modes of vesicles dynamics, and allowed us to use FRAP to measure the vesicle diffusion coefficient ($\sim 0.29 \mu m^2 s^{-1}$). While we assumed that the VAMP72 fluorescent marker exclusively labeled secretory vesicles, it remains a possibility that the marker could have labeled a small fraction of the Golgi or ER. However, our Numbers and Brightness (N&B) analysis shows that the vast majority of pixels in the images have a brightness distribution consistent with the marker labeling a single species (Figure 3.10C). In addition, fluorescence labeling of the ER and Golgi (Furt et al., 2012) did not match the fluorescence intensity distribution found with VAMP72. Furthermore, the vesicle diffusion coefficient we measured is
in agreement with previously measured pollen tube vesicle diffusion coefficients (Bove et al., 2008; Kroeger et al., 2009), and measurements of the viscosity of the moss cytoplasm (Kingsley, Bibeau et al., see section 2).

Our quantitative estimate of the vesicle diffusion coefficient, the number of vesicles (through N&B analysis of VAMP72), and the kinetics of vesicle fusion ($mK_{on}$) allowed us to build a model to describe diffusion-based growth with relevant parameters from moss. While the variability in number of VAMP72 molecules on a vesicle can contribute to the observed intensity fluctuations, we considered this effect to be negligible since our measured concentration of vesicles is in agreement with EM estimates (Mccauley and Hepler, 1992). We used this concentration to solve the flux equation at the cell tip, and determined an estimate for the kinetics of vesicle fusion, $mK_{on}$. To explore how the reaction kinetics observed during normal cell growth limit diffusion based cell growth, we assumed $mK_{on}$ is constant and independent of the presence of F-actin. With these parameters used as inputs, we built and solved a comprehensive model of diffusion-based cell growth in a realistic moss geometry. It is important to note that this model is a theoretical proof used to illustrate limitations that highlight the importance of F-actin, and is not expected to match the physiological conditions in latrunculin B treated cells. Nevertheless, our results predict that diffusion-based transport is slower than normal cell growth and heavily dependent on the size of the active region of exocytosis.
Finally, with driselase treatments, we found that the effective length scale for the region of greatest extensibility (maximum secretion) was on the order of a few microns and located at the cell tip. This analysis holds as long the cell wall extruded because of an increased extensibility, and not because of increased stresses resulting from the intrinsic geometry of the cell. Based on the shape of the cell wall, and the assumption that the cell wall is a thin sheet, one can show that the maximum stresses on the cell wall are not at the regions of rupture (Campàs and Mahadevan, 2009) (see section 1.4 for more details). For thin walled shells under uniform pressure, it has been shown that the curvature of the cell wall

**Figure 3.13.** F-actin must overcome the limiting factors in polarized cell growth. A) Illustration of limiting factors in cell growth. Without F-actin, our measured vesicle diffusion, vesicle concentrations, reaction kinetics \(mK_{on}\), and the active secretion zone size would result in a significantly slower polarized growth. B) With F-actin the cell can overcome these measured limitations and sustain cell growth.
is inversely proportional to stress (Campàs and Mahadevan, 2009). Since the shank of the cell is less curved than the tip, greater stresses are predicted at the shank; therefore the observed cell rupture is most likely a result of increased extensibility at the tip. Additionally, these measurements for the size of the exocytic zone are consistent with the localization of myosin XI at the cell tip (Vidali et al., 2010).

Although we built a tip-growth model that quantitatively highlights the necessity for an active transport mechanism in polarized growth, we cannot rule out the possibility that other factors could limit polarized growth. For example, the F-actin could be necessary for maintaining the active region of exocytosis. Specifically, F-actin could facilitate the transport and polarization of the receptors required in vesicle fusion. However, elucidating such potential limitations is left for future work. Importantly, we have shown and quantified specific reasons why active transport is necessary in polarized growth. These analyses and modeling approaches are likely to apply to other tip growth systems in plants such as root hairs and pollen tubes.

3.5 Methods

3.5.1 Measuring Moss Growth Rates
The moss cell line used for measuring growth rates was 3mEGFP-VAMP72, and was transformed as done in (Furt et al., 2013) the gene used corresponds to accession number Pp3c4_13580V3.1 (Phytozome 12). Moss samples were cultured using the protocol described previously (Vidali et al., 2007). Microscope samples were prepared as described previously (Furt et al., 2013). To stop growth,
liquid medium (PpNO₃) with 200μL of 5 μM of latrunculin B was pipetted directly on top of the growing cells. Samples were imaged on a Leica TCS SP5 confocal microscope with a 63X objective and a numerical aperture of 1.4. The argon laser was set to 25% power and the 488 nm laser line was set to 10% power. The emission bandwidth was set between 499 – 546 nm, and a hybrid detector was used to acquire images. Kymographs and growth rates were constructed using ImageJ.

3.5.2 Experimental FRAP Acquisition, Processing, and Analysis
The moss cell line used for FRAP was a 3mCherry-VAMP72 3mEGFP-myosin XIa double line previously published by (Furt et al., 2013). Moss samples were cultured using the protocol described previously (Kingsley, Bibeau et al., see section 3.5.2). Samples were prepared in QR-43C chambers (Warner Instruments) and perfused with 5 μM latrunculin B. Samples were imaged on a Leica TCS SP5 scanning confocal microscope using the Leica FRAP wizard. A 63X objective with a 1.4 numerical aperture was used. The pinhole was set to 2 airy disks and the camera zoom was 9. To visualize the 3mCherry-VAMP72 cell line developed in (Furt et al., 2013), the DPSS 561 nm laser was turned on and the 561 nm laser line was set to 10%. The laser scanning speed was set to 1400 Hz with the bi-directional scanning. The emission bandwidth for 3mCherry was set between 574 and 646 nm. Images were acquired at 256 × 256 pixel resolution, with a bit depth of 12 bits.

Following image acquisition, images were subjected to several processing steps. First experimental TIFF stacks were analyzed with an ImageJ macro that
tracked the photobleaching Region Of Interest (ROI), performed background subtraction, and normalized the mean ROI to pre-bleach ROI intensities. Image acquisition photobleaching was measured by tracking fluorescence intensity over time while imaging cells at the same sampling rate used during FRAP experiments. This fluorescence time trace was fit to a piecewise exponential. To correct for acquisition photobleaching and reversible photobleaching, normalized FRAP experimental ROI intensities were divided by this piecewise exponential (Figure 3.3). All untreated curves were normalized to the mean pre-bleach shank intensity to illustrate changes in fluorescence intensity relative to the shank vesicle concentration. Recovery curves from latrunculin B treated cells, at the tip and shank, were normalized to their mean pre-bleach intensities at their respective locations.

For spatial analysis of fluorescence recovery, experimental images were analyzed as follows. First an image cropping macro was used to extract FRAP ROIs. Cropped ROIs were then normalized to the mean of the pre-bleach ROIs and averaged across experimental replicates. To correct for the limited accessible volume at the cell tip, we fit an exponential decay to a line scan through the cropped prebleach ROIs. We then divided each ROI during fluorescence recovery by this exponential decay (Figure 3.7). For more details on experimental FRAP acquisition and processing see, (Kingsley, Bibeau et al., see section 2.5).

After ROI processing, we spatially sub-sampled the ROIs with an angular sector that was rotated 360 degrees about the ROI (Figure 3.2B and 3.3B). To reduce noise, we averaged about the horizontal axis of the ROI; this produced an
intensity profile from 0 – 180 degrees.

3.5.3 Measuring Confocal Imaging and FRAP Parameters
Since our particle-based FRAP simulation considers the imaging and photobleaching properties of the confocal microscope, we measured the Point Spread Function (PSF) of our imaging system, and the photobleaching properties of 3mCherry-VAMP72 (Kingsley, Bibeau et al., see section 2.5.8 for more details on the simulation inputs and parameter estimates). To determine the PSF of our imaging system, we used the PS-Speck Microscope Point Source Kit P7220 (Invirtogen). Bead images (Figure 3.6) were deconvolved with a box car function equal to the width of the bead. This ensured that the experimental images represented a true point source. The deconvolved PSF was then fit to a three-dimensional squared Gaussian beam (Figure 3.6) and used for imaging in the simulation (Kingsley, Bibeau et al., see section 2.5.4). Based on these fits, we found that the minimum beam width, $w_0$, and the Rayleigh range, $z_R$, were 520 and 500 nm, respectively.

To measure the photobleaching properties of the beam, we fixed cells expressing 3mCherry-VAMP72 with formaldehyde (Fritzsche and Charras, 2015). For fixation, a solution of 100 mM HEPES with 2% formaldehyde at pH 7, was perfused into QR-43C chambers (Warner Instruments) for 30 minutes (Vidali et al., 2007). Fixed cells were subjected to a $1 \times 6 \, \mu m$ rectangular photobleaching region of interest (Figure 3.6C, solid white rectangle) and imaged in the same plane as the photobleach. A rectangular ROI was chosen because it is composed of confocal scans of equal length; which permits fluorescence averaging along the
long axis of the rectangle. A line scan was created by collapsing a $4 \times 8 \mu m$ rectangular region including the photobleached ROI (Figure 6, dashed yellow rectangle) into a one-dimensional profile. After photobleaching, we fit the photobleaching function described by (Kingsley, Bibeau et al., see section 2.5.5) to the line scan. This function characterizes the horizontal line scan of a Gaussian beam as it photobleaches across a fixed distance. As shown previously (Braeckmans et al., 2006), we found that the photobleaching Gaussian beam waist was two-fold larger than the imaging beam waist at 920 nm. This fit also yielded a photobleaching proportionality constant, which was used as a starting point in fluorescence recovery fitting routines to measure VAMP-72 vesicle diffusion. For more details on simulating confocal photobleaching see, (Kingsley, Bibeau et al., see section 2.5.5) and for general use we developed a Digital Confocal Microscopy Suite (DCMS), which can be found at http://dcms.tuzelgroup.net.

3.5.4 FRAP Parameter Minimization
To determine the simulation parameters that best characterize the recovery curves for VAMP72-vesicles in latrunculin B treated cells, a minimization routine was used similar to the one by (Kingsley, Bibeau et al., see section 2.5.8). Averaged experimental recovery curves $R_{exp}^{exp}(t)$ at the tip and shank were characterized into classes $R_T^{exp}(t)$ and $R_S^{exp}(t)$, respectively. Averaged simulation recovery curves were characterized in the same way to produce $R_T^{sim}(t)$ and $R_S^{sim}(t)$. These two classes of simulation recovery curves were generated in a two-dimensional parameter space for the parameters $D$ and $K$, where a range of each parameter was simulated ($D = 0.1 - 0.5 \mu m^2 s^{-1}$ and $K = 0.01 - 0.03$). Here $D$ is the diffusion
coefficient and $K$ is the bleaching proportionality coefficient. The bleaching proportionality coefficient $K$ measured during fixation was used only as a reference in generating the range for possible values of $K$. This was done because formaldehyde treatment was found to affect fluorophore bleaching properties at the concentrations used. Unlike the approach from Kingsley, Bibeau et al., (see section 2.5.8) only two parameters were used here because the photobleaching width, $w_0$, was measured with formaldehyde treated cells. The recovery curve for each parameter pair was stored in a Matlab (MathWorks, Natick, MA) data structure. Best fit simulation parameters for the averaged experimental recovery profiles at the tip, $R_{T}^{exp}(t)$, and shank, $R_{S}^{exp}(t)$, were found by reducing the sum of squared differences between the curves. Averaged tip and shank recovery curves can be represented as $R_{T}^{sim}(t)_{D,K,w_0}$ and $R_{S}^{sim}(t)_{D,K,w_0}$, respectively.

To account for reversible bleaching we imposed a correction on all simulated recovery curves (Sinnecker et al., 2005; Mueller et al., 2012; Morisaki and McNally, 2014) (Kingsley, Bibeau et al., see section 2.5.7). During intentional photobleaching, some fraction of the bleached molecules, $\alpha$, convert back to an unbleached state at a given rate, $R(t)$. We used $\alpha$ and $R(t)$ that were previously measured for mCherry. Then using the relationship previously devised, $I_M(t) = I_{FRAP}(t) + \alpha R(t) I_{FLAP}(t)$, we corrected the simulated recovery curves (Mueller et al., 2012). Here $I_M(t)$ is the resultant fluorescence recovery that incorporates reversible bleaching, $I_{FRAP}(t)$ represents the fraction of the fluorescent recovery due to movement of unbleached fluorophores and $I_{FLAP}(t)$ represents the motion of the reversibly photobleached molecules. After correction the averaged
simulation tip and shank recoveries become, \( R^{\text{sim}}_T(t)_D^{\text{rev}}_{w_0} \) and \( R^{\text{sim}}_S(t)_D^{\text{rev}}_{w_0} \), respectively, where the superscript \( \text{rev} \) indicates reversible bleaching correction.

Following this correction, best fit diffusion coefficients for VAMP72-vesicle experiments, were found with the following argument minimization,

\[
\text{argmin}_{D_v,K_t,K_s} \left[ \sum_{t>0} (R^{\text{exp}}_T(t) - R^{\text{sim}}_T(t)_D^{\text{rev}}_{w_0})^2 + \sum_{t>0} (R^{\text{exp}}_S(t) - R^{\text{sim}}_S(t)_D^{\text{rev}}_{w_0})^2 \right] \quad \text{(Eq. 3.9)}
\]

In this minimization, experimental recoveries at the cell tip and shank were compared to their corresponding simulations to find the parameters \( D_v, K_t \), and \( K_s \). Here, the subscript \( v \) denotes VAMP72-vesicles. As shown in Eq. (3.9), the diffusion coefficient \( D_v \) was shared between the tip and shank. To improve the argument minimization, two unshared bleaching proportionality coefficients were used at the tip \( K_t \) and shank \( K_s \) (Kingsley, Bibeau et al., see section 2.5.7).

Although using two unshared bleaching probabilities improves the argument minimization, a shared bleached probability for the tip and shank did not appreciably change the mean value of our measured diffusion coefficient, \( D = 0.45 \pm 0.04 \, \mu\text{m}^2\text{s}^{-1} \). This measurement has confidence intervals that overlap with the intervals of our VAMP72-vesicles diffusion coefficient (\( D = 0.29 \pm 0.14 \, \mu\text{m}^2\text{s}^{-1} \)) and does not change our conclusions. Similarly, we found that the ignoring reversible photobleaching would have had little impact on our measured diffusion coefficient (\( D = 0.32 \pm 0.18 \, \mu\text{m}^2\text{s}^{-1} \)).

To determine the error associated with the measured parameters, we used the Monte Carlo simulation as described in detail in (Kingsley, Bibeau et al., see
section 2.5.8)(Motulsky and Christopoulos, 2004). In brief, we generated new experimental and simulated recovery curves via Monte Carlo simulation by sampling from a normal distribution for each condition. The mean and standard deviations of this normal distribution were taken from the mean and standard deviations of each point in the corresponding recovery curve. We then performed the argument minimization described in Eq. (3.9) on the newly generated curves to find new values for the parameters, $D_\nu$, $K_t$, and $K_s$. We then repeated this process to produce a distribution of these parameters. We used two standard deviations from each distribution to represent the error of our measured parameters such that,

$D_\nu = 0.29 \pm 0.24 \mu m^2 s^{-1}$, $K_t = 0.02 \pm 0.004$, $K_s = 0.0125 \pm 0.004$.

3.5.5 Comsol Cell Growth Model

Cell growth models were generated using the Comsol Multiphysics (Comsol Inc, Stockholm, Sweden) modeling software using the “three-dimensional transport of dilute species” interface with one species. To achieve the steady state solution of our growth model, the “stationary study” option was selected. We model the cell as a cylinder of length $10 \mu m$ and radius $6 \mu m$, capped by a hemisphere. To simulate the active region of exocytosis, we induced a flux, consistent with Eq. (3.8), through the cell membrane at the cell tip. To simulate the uniform production of vesicles, we imposed a homogenous reaction rate. All simulations were run with a mesh setting of “extremely fine” in the software. To determine the concentration profiles required to maintain wild type growth, for the various secretion sizes, we set the production term to match the desired growth rate. We then modified this production term to match the experimentally measured shank concentrations and
reported the growth rate. Vesicle concentration profiles were then exported as text files, and visualized in Matlab.

3.5.6 Brightness and Numbers Analysis
Moss cultures and microscope samples were prepared as described in measuring moss growth rates. Samples were imaged on a Leica TCS SP5 confocal microscope with a $63 \times$ objective with a numerical aperture of 1.4. The argon laser was set to 25\% power and the 488 $nm$ laser line was set to 20\% power. The emission bandwidth was set between 499 – 546 $nm$ and a hybrid detector in photon counting mode was used. Images were scanned at 700 $Hz$. Six latrunculin B-treated cells were imaged at both the shank and the cell tip within a $6 \times 6 \mu m$ area, and a pixel resolution of $512 \times 512$. Image stacks were analyzed in Matlab (MathWorks, Natick, MA) as previously described (Digman et al., 2008).

To conduct our analysis, we collected confocal image time series at the tip and shank of latrunculin B treated cells expressing 3mEGFP-VAMP72 (Figure 3.10A). We assumed that all the VAMP72s on a vesicle equally contribute to the brightness of a vesicle because the size of a vesicle is consistent with a diffraction limited spot (Lancelle and Hepler, 1992). To ensure that detector shot noise and VAMP72-vesicle number fluctuations were the primary contributors to intensity fluctuations, all images were acquired with a hybrid detector in photon counting mode (Dalal et al., 2008; Digman et al., 2008). The intensity fluctuations over time for each pixel were calculated and used to determine the brightness and number of molecules within each pixel (Figure 3.10). We then manually filtered the pixels by their apparent brightness and intensity. This was done to remove background
pixels and anomalously bright pixels (with brightness value $B > 2$) (Figure 4.10C). To convert the number of molecules within a given pixel to a concentration, the number of molecules must be divided by the volume of the Point Spread Function (PSF). To obtain the apparent volume of the PSF, we used our particle-based confocal simulation (Kingsley, Bibeau et al., see section 3.5.6) with our measured PSF beam width and Rayleigh range as input parameters. These parameters were measured in the same way as 3mCherry (Figure 3.6). With the particle-based confocal simulation we then generated an array of vesicle concentrations, and used a calibration curve to convert the number of molecules per pixel to the known concentrations (Figure 3.10D). From the calibration curve, we were able to determine the volume of our PSF given our measured experimental parameters. This PSF volume was within 20% error of theoretical PSF volumes (Moens et al., 2011).
3.5.7 Driselase Treatment on the Cell Wall
Moss cultures were prepared as described in measuring moss growth rates. Microscope preparations were made on the bottom of 60X15 mm petri dishes. 10 ml of PPN03 with 1% agar was pipetted into the dish. Six 1 ml pipette tips were placed face up onto the dish. Once solidified, the pipette tips were removed and 50 μL of PPN03 with 1% agar was pipetted into the newly made holes. A single moss colony was then placed into one of the holes. Then a 200 μL solution of 8% mannitol and 2% driselase was pipetted into the hole. The cells were imaged using a Zeiss Axio Observer inverted microscope with a 20 × objective. Images were acquired with a Photometrics Cool Snap Camera with the Pixelfly software. Images were taken every 0.1 seconds.

Images were analyzed with Matlab (MathWorks, Natick, MA). Cell edges were detected using the canny edge detection algorithm with the function edge. Rupture points and positions were found manually by selecting points on the image. Polynomial fitting was used to estimate relevant arc lengths.
4 FRAP and VAEM Reveal Fast Interactions Between Myosin XIa, VAMP72-labeled Vesicles, and Filamentous Actin

4.1 Abstract
The apical actin cytoskeleton and active membrane trafficking machinery are essential in driving polarized cell growth. Nevertheless, the coordinated interaction between these systems has yet to be elucidated in plants. Specifically, myosin XIa has yet to be shown to interact with its cargo, or filamentous actin in vivo. To better understand these potential interactions, we used Variable Angle Epifluorescence Microscopy (VAEM) in Physcomitrella patens protoplasts to demonstrate that myosin XIa and VAMP72-labeled vesicles localize in time and space for periods lasting on the order of seconds. Using VAEM of P. patens caulonemal and protoplast cells in conjunction with Hidden Markov Modeling (HMM), we also showed that myosin XIa and VAMP72-labeled vesicles exhibit actin dependent directed transport. Furthermore, we observed active transport run lengths in caulonemata to have an end-to-end distance of $0.85 \pm 0.1$ and $0.70 \pm 0.08 \, \mu \text{m}$ that both lasted less than $0.55 \, \text{ms}$ for myosin XIa and VAMP72-labeled vesicles, respectively. With Fluorescence Recovery After Photobleaching (FRAP), we showed that the directed transport of myosin XIa and VAMP72-labeled vesicles, at the growing cell tip, is actin dependent and consistent with the speeds measured with VAEM. Using quantitative particle tracking and finite element modeling of reaction-diffusion FRAP, we estimated that myosin XIa and VAMP72-labeled vesicles have a dissociation constant, $K_d$ of $\sim 5-50 \, \mu \text{M}$. This work shows in
vivo that myosin XIa and VAMP72-labeled vesicles have a weak interaction with each other, and that they exhibit short processive transient interactions with filamentous actin. These low affinities are likely to be important for the dynamic oscillations observed at the cell apex, and may be vital for flexible regulation and recycling of the exocytosis machinery; while simultaneously promoting the vesicle focusing necessary for cell wall expansion.

4.2 Introduction
Polarized cell growth, a mechanism by which plasma membrane and cell wall material are deposited to a defined region of the cell, is widespread across the plant kingdom. As a result, plant cells exhibit a large variety of different shapes well-designed to achieve different functions (Geitmann and Ortega, 2009; Szymanski and Cosgrove, 2009). Tip growth is an extreme form of polarized cell growth where the cell expands only in one direction generating elongated cells that can be hundreds of μm long (Hepler et al., 2001; Menand et al., 2007). This specialized form of growth is particularly well suited for pollen tubes to reach the ovule during sexual reproduction (Hepler et al., 2001; Cole and Fowler, 2006), for root hairs to uptake nutrients and water from the soil (Gilroy and Jones, 2000; Hepler et al., 2001), and for bryophytes to colonize on land (Kenrick and Crane, 1997; Heckman et al., 2001). This process has been extensively studied in pollen tubes from lily and tobacco, root hairs from Arabidopsis and protonemal cells from Physcomitrella patens. It is well known that it requires a dynamic actin cytoskeleton network as well as active membrane trafficking machinery (Kenrick and Crane, 1997; Gilroy and Jones, 2000; Heckman et al., 2001; Hepler et al., 2001; Cole and
Fowler, 2006). To support cell growth, modeling evidence suggests that endomembrane vesicles must be transported and exocytosed to bring cell wall material to the tip of the elongated cells (Bibeau et al., 2017). Because the wall material required for growth far exceeds the necessary membrane material, endocytosis occurs to recycle the excess plasma membrane. However, how these different types of machineries are coordinated with the actin cytoskeleton is still unknown. To better understand how the tip growing cells self-organize their cytoplasmic components to achieve and maintain polarized growth, it is critical to determine the function of each component separately as well as their interactions.

Several studies have shown that the actin-based molecular motor myosin XI is required for tip growth in plants. Silencing of the two myosin XI genes—Myosin XIa and b—in the moss *Physcomitrella patens* resulted in round cells where tip growth was completely abolished (Vidali et al., 2010). Dominant negative inhibition and gene knockout approaches suggested that among the 13 isoforms of myosin XIa present in *Arabidopsis*, myosin XI-K is a primary contributor for root hair elongation by tip growth (Ojangu et al., 2007; Peremyslov et al., 2008; Prokhnevsky et al., 2008; Park and Nebenfuhr, 2013); while myosin XIC1 and c2 are important for pollen tube growth (Madison et al., 2015). In the last decade, it has been well established that myosin XIs are responsible for the motility of large organelles in higher plants, even though their relative contribution and interdependency relationships are not fully understood (Avisar et al., 2008; Peremyslov et al., 2008; Prokhnevsky et al., 2008; Sparkes et al., 2008; Avisar et al., 2009; Peremyslov et al., 2010, 2010; Ueda et al., 2010; Avisar et al., 2012;
Vick and Nebenfuhr, 2012; Madison and Nebenfuhr, 2013; Griffing et al., 2014; Henn and Sadot, 2014). However, no correlation has been found between the root hair elongation phenotype and the organelle motility in the myosin xi-k knockout mutants (Prokhnevsky et al., 2008). In addition, the apical localization of myosin XIa in moss caulonemal cells does not correlate with the localization observed for the large organelles (Vidali et al., 2010; Furt et al., 2012). Therefore, the function fulfilled by myosin XIa that is essential to achieve and/or maintain tip growth in plants is still unknown.

Numerous studies have reported that myosin Vs, which are the homologues of myosin XIs in animals and yeast, are involved not only in large organelle motility but also in secretory, endocytic and recycling pathways via their ability to transport endomembrane vesicles (Pruyne et al., 1998; Schott et al., 1999; Lapierre et al., 2001; Ohyama et al., 2001; Rodriguez and Cheney, 2002; Volpicelli et al., 2002; Fan et al., 2004; Yan et al., 2005; Lise et al., 2006; Nedvetsky et al., 2007; Roland et al., 2007; Li and Nebenfuhr, 2008; Hammer and Sellers, 2012). Recent evidence suggests that myosin XIs could be involved in endomembrane vesicle transport in plants. Results from co-localization with vesicle markers (RabA4b and SCAMP2), fluorescence recovery after photobleaching, and biochemical co-fractionation with vesicle (RabA4b) and exocytic (Sec6) markers inferred that myosin XI-K is associated with endomembrane vesicles in both leaf cells and root hairs in Arabidopsis (Peremyslov et al., 2012; Park and Nebenfuhr, 2013; Rybak et al., 2014). Using confocal microscopy combined with fluctuation cross-correlation analyses, we previously demonstrated that myosin XIa and VAMP72, an
endomembrane vesicle marker, co-localize at the cell apex and are synchronized during tip growth (Furt et al., 2013). Surprisingly, we also showed that apical myosin XIa precedes F-actin during polarized growth of *P. patens* caulonemal cells (Furt et al., 2013). Pharmacological approaches using latrunculin B to depolymerize the actin filaments, further showed that myosin XIa stays associated with the VAMP72 marker at the apex of moss cells after disruption of the actin network (Furt et al., 2013). In addition, we observed the emergence of ectopic clusters of myosin XIa associated with VAMP72-labeled vesicles, which were then propelled through the cell via an actin-dependent manner when the actin network self-reorganizes (Furt et al., 2013).

Together, these results suggest the existence of a mechanism where myosin XI-associated endomembrane structures could organize the actin polymerization machinery. However, the apex of plant tip growing cells being densely occupied by vesicles (Parton et al., 2001; Preuss et al., 2004; de Graaf et al., 2005; Ovecka et al., 2005; Bove et al., 2008; Zonia and Munnik, 2008; Kroeger et al., 2009; Szumlanski and Nielsen, 2009), and the resolution of confocal laser scanning microscopes being diffraction limited, make it challenging to visualize single vesicles (~80 nm in diameter) in plant tip growing cells to uncover this mechanism. Therefore, the ability of myosin XIa to transport endomembrane vesicles and coordinate this transport with the actin polymerization machinery remains to be demonstrated, and whether this is the essential function fulfilled by myosin XIa to achieve polarized growth remains to be proved. Furthermore, there are no quantitative in vivo estimates of these binding interactions in plants.
Specifically, the average on and off-rates of myosin XI and its cargo are not known, furthermore myosin XI run lengths and off-rates on actin filaments remain unknown. For this reasons, any quantitative insight into these interactions would advance the fields understanding of how the cytoskeleton behaves and would facilitate future modeling of the cytoskeleton in polarized growth.

In this study, we used variable angle epifluorescence microscopy (VAEM) to image single endomembrane vesicles. We show that both myosin Xla and VAMP72 co-localize at the cortex of moss cells to highly dynamic punctate structures, whose motility depends on actin filaments. With particle tracking and Hidden Markov Modeling, we characterized the weak transient interactions between myosin XI, VAMP72-labeled vesicles, and filamentous actin. We then use fluorescence recovery after photobleaching (FRAP) (McNally, 2008; Loren et al., 2015) to demonstrate that myosin Xla recovers at the cell apex in an actin-dependent manner. Our results are consistent with the hypothesis that myosin Xla coordinates the traffic machinery and the actin network dynamics to maintain polarized growth in moss cells.
4.3 Results

4.3.1 Myosin XIa and VAMP72 Labeled Highly Dynamic Punctate Structures at the Cortex of Moss Cells

We previously showed that myosin XIa fluctuations anticipate F-actin’s at the apex of caulonemal cells while myosin XIa levels fluctuate in identical phase with VAMP72, an endomembrane vesicle marker. To explain this, we proposed a model where myosin XIa, via its association with endomembrane vesicles, coordinates the vesicular traffic and the F-actin polymerization-driven motility at the cell apex, to maintain polarized cell growth (Furt et al., 2013). To further decipher the mechanisms underlying such interactions, we used VAEM which can provide the resolution necessary to image single endomembrane vesicles (Konopka and Bednarek, 2008). We found, as expected, that VAMP72 labeled highly dynamic small punctate structures at the cortex of both protoplasts and apical caulonemal cells (Figure 4.1A and 4.1B respectively, top panels). This is consistent with localization to endomembrane vesicles, and observations reported in protoplasts.

![Figure 4.1](image.png)

**Figure 4.1.** VAMP72 and myosin XIa localize to punctate structures at the cell cortex of moss cells. A) Two representative images of cortical localization of 3mCherry-VAMP72 and 3mEGFP-myosin XIa in protoplasts acquired using VAEM. Scale bars are 4 μm. B) Two representative images of cortical localization of 3mCherry-VAMP and 3mEGFP-myosin XIa in apical caulonemal cells acquired using VAEM. Scale bars are 2 μm.
from *Arabidopsis* suspension cultured cells using the same probe ((Uemura et al., 2004). We also showed that Myosin Xla localizes to similar dynamic punctate structures at the cortex of both protoplasts and apical caulonemal cells (Figure 4.1A and 4.1B respectively, bottom panels).

### 4.3.2 Myosin Xla Exhibits a Weak Interaction with VAMP72-labeled Vesicles in Moss Protoplasts

To determine if myosin Xla and VAMP72 are associated with the same membranes, we simultaneously imaged myosin Xla-labeled structures and VAMP72-labeled vesicles, and we recorded time-lapse series to observe their respective trajectories in protoplasts. We then tracked the movement of the punctate structures labeled by myosin Xla and VAMP72 using the TrackMate plugin from ImageJ (see Methods). Using an approach similar to (Deschout et al., 2013) we used an automated analysis to determine if the myosin Xla and VAMP72-labeled vesicles exhibited correlated movement within the same area. In this approach we used a minimal contact radius and a sliding correlation window to determine if two particles from opposite channels exhibited correlated movement. For each given window size, the frequency of VAMP72-labeled trajectories was calculated by dividing all the VAMP72-labeled trajectories by the total VAMP72-labeled trajectories (Figure 4.2A and Methods). We found that a fraction of myosin Xla-decorated structures partially co-localize —in time and space— with VAMP72-labeled vesicles at the cell cortex in moss protoplasts (Figure 4.2A). We observed that at least 20% of the VAMP72-labeled trajectories exhibited some co-localization during a total period of 400 ms, and at least 15% during a total period
of 1 s (Figure 4.2B). These co-localization frequencies are significantly higher than those obtained by randomly shuffling the observed trajectories (Figure 4.2B, Random Shuffle). The reduction in localization frequency at increasing window sizes is the result of increased stringency for co-localization at bigger window sizes. Examples shown in Figures 4.2C and 4.2D demonstrate these co-localized trajectories between myosin XIa-labeled structures and VAMP72-labeled vesicles. To examine the total co-localization time of both myosin XIa and VAMP72-labeled vesicles, we divided the total number of co-localization events by the total number of identified events. Of all identified and tracked myosin XIa, we found that ~4.2% of it was localized to vesicles; we also found that 10% of the identified VAMP72-labeled vesicles were co-localized with a myosin XI. Although these percentages may seem low, it is important to note that they result from the co-localization analysis that requires spatial co-localization in time and space. Because of the stringency of this analysis, these measurements are well above random co-localization events (Figure 4.2B). This result is consistent with the hypothesis that myosin XIa associates with endomembrane vesicles.

To gain insight into the potential myosin XIa vesicle interaction, we modeled this process as the reversible reaction, \( U + S \rightleftharpoons B \). Here \( U \) represents the concentration of free myosin XIa in the cytoplasm, \( S \) represents the concentration of available vesicle receptors for myosin XIa, and \( B \) represents the concentration of myosin XIa bound to a vesicle receptor. Assuming our observations take place at equilibrium, the following relationships can be used to relate the reaction
constants and the observed equilibrium concentrations (Sprague and McNally, 2005; Kang and Kenworthy, 2008; Kang et al., 2010),

$$\frac{K_d}{S_{eq}} = K_d^* = \frac{k_{off}}{k_{on}} = \frac{U_{eq}}{B_{eq}} \quad (Eq. \ 4.1)$$

$$B_{eq} = Myo_{total} - U_{eq} \quad (Eq. \ 4.2)$$

$$S_{eq} = Ves \times Sites - B_{eq}. \quad (Eq. \ 4.3)$$

Here $Myo_{total}$ is the total concentration of myosin XIa, the subscript $eq$ denotes equilibrium concentration, $Ves$ is the vesicle concentration, $Sites$ is the number of receptors on a single vesicle, $K_d^*$ is the effective dissociation constant, $K_d$ is the dissociation constant, $k_{off}$ is the reaction off-rate, $k_{on}^*$ is the effective on-rate, and $k_{on}$ is the reaction rate. If we have the resolution to do single particle tracking and assume that our tracking of myosin XIa isn’t biased towards bound or unbound myosin XIa, the observed 4.3% bound fraction of myosin XIa yields an effective dissociation constant of $K_d^* \sim 22$ (Eq. 4.1).

To estimate the off-rate for this interaction, we examined the duration myosin XIa resided on a vesicle. Fitting the observed residence times to an exponential decay of the form, i.e.,

$$n = \frac{n(t_0)}{e^{-k_{off}t_0}} e^{-k_{off}t} \quad (Eq. \ 4.4)$$

yields an off rate of $k_{off} \sim 2 \text{ s}^{-1}$ (Figure 4.3). Here $n$ is the number of molecules bound after the elapsed time $t$, and $t_0$ is the minimum detection required to characterize a particle as bound. $t_0$ results from the frame rate (50 ms) and the window size used in the co-localization algorithm. This off-rate is an upper limit
estimate because we cannot be certain when each association began and movement out of the evanescent field reduces our observed association times.

To establish an estimate of the reaction on-rate, and obtain a true dissociation constant, we used numbers and brightness (N & B) to estimate the total concentration of myosin Xla (Figure 4.4) (Digman et al., 2008; Bibeau et al., 2017). Briefly, this method utilizes the fact that intensity fluctuations, in a confocal image acquired with a hybrid detector, are the result of Poisson distributed particle fluctuations and Poisson distributed detector shot noise. To remove myosin Xla and filamentous actin-binding we depolymerized the actin cytoskeleton with 20 μM latrunculin B. If we assume that the intensity fluctuations due to the number of myosin Xla on a vesicle are negligible we obtain a concentration of 114 ± 14 nM. Assuming that caulonemata have roughly the same myosin Xla concentration as protoplasts, that the number of receptors on a vesicle are within the range Sites = 4-40, and that the concentration of vesicles is 60 nM (Bibeau et al., 2017), we find the dissociation constant and on-rate to be within the following ranges, $K_d = 5 - 50$ μM and $k_{on} = 10 - 100 \mu M^{-1}s^{-1}$. Taken together, our particle tracking data suggest that there is a weak interaction—with μM affinity—between myosin Xla and VAMP72-labeled vesicles in protoplasts.
Figure 4.2. Myosin XIa co-localizes and moves with VAMP-labeled vesicles at the cell cortex. A) Co-localized trajectories of 3mCherry-VAMP and 3mEGFP-myosin XIa detected at the cortex of one protoplast over a total period of 100 s. Blue numbers and trajectories indicate sample co-localization events in (C) and (D). Scale bar 10 µm. B) Frequency of 3mCherry-VAMP72 trajectories that contain co-localization with 3mEGFP-myosin XIa detected in protoplasts (black square) or randomly generated by a simulation (red circle). x-axis represents the minimum required number of frames two particles have to be moving together to be classified as co-localized. N=3 cells. C) and D) Selected images of the co-localized trajectories 1 and 2, respectively, highlighted in cyan in (A). 3mCherry-VAMP and 3mEGFP-myosin XIa are shown in the top two panels and the merged image in the third one with VAMP-labeled vesicles in red and myosin XIa in green. Images from one transgenic line were simultaneously acquired from protoplasts using VAEM at 100 ms intervals for the complete time series. Selected images are shown with the time from the series indicated at the top. Scale bar 2 µm. Maximum projections of 26 frames from each corresponding time series shown in the right panel. Scale bar 2 µm.
Figure 4.3. Observed myosin XIa vesicle off-rate $k_{off}$. The number of myosin XIs observed on a vesicle in protoplasts for a given time (blue line) and the corresponding best fit to Eq. 4.4 (red dotted line).

Figure 4.4. Measuring myosin XIa concentration with numbers and brightness (N&B). A) Time series of caulonema cells expressing myosin XI-3mEGFP following latrunculin B treatment. Green scale bar is 10 μm. B) Example intensity trace for a given pixel location in (A). C) Brightness and intensity distribution for all the pixels in the time series shown. Color map indicates frequency, where dark blue indicates low frequencies and yellow indicates high frequencies.
4.3.3 Myosin XIa-associated Structures and VAMP72-labeled Vesicles Exhibit Co-localized Active Motion in Protoplasts

We observed that several punctate structures labeled with both myosin XIa and VAMP72 move in a linear manner, as indicated by the maximum projections in the examples shown in Figure 4.2C and 4.2D. Also, as shown in Figure 4.2D, we were also able to detect several structures moving in succession along the same linear trajectory. Together with the fact that myosin XIa is an actin-based motor, these results strongly suggest that the motion of the endomembrane vesicles labeled by both myosin XIa and VAMP72 occurs on actin filaments.

To test whether VAMP72-labeled vesicles are transported by myosin XIa on actin filaments, we first simultaneously imaged myosin XIa and F-actin. We found that myosin XIa-labeled structures co-localize and move along cortical actin filaments in moss protoplasts (Figures 4.5A and 4.5B).
To more quantitatively analyze how myosin XIa and VAMP72 together associate with F-actin—without the need for tracking individual actin filaments—we used a hidden Markov model, HMM (see Methods for more details) (Rabiner, 1989). Briefly, an HMM is a statistical model for determining the likelihood, that at a given
time, a system is in some unobserved state based on a known observable variable. As previously shown (Roding et al., 2014), an HMM can be applied to intracellular particle tracking data to determine the likelihood that, at a specific point in a given trajectory, a particle is either in a Brownian state or an active transport state, Figure 4.6A. Importantly, this model does not require fluorescently labeling the actin cytoskeleton or tracking individual actin filaments in crowded environments. For this reason, this analysis does not introduce filament-tracking error, manual tracking biases, and permits simultaneous tracking of myosin XIa and VAMP72-vesicles.

Figure 4.6. Hidden Markov model explained. A) Example hidden sequence H and the corresponding observed sequence Q. In the hidden sequence green As correspond to the active transport state and red Bs correspond to the Brownian state. In the observed sequence, black Fs correspond to forward moves and black Rs correspond to backward moves. B) Example of trajectory conversion to forward and backward moves. Step 1) indicates how the angle \( \theta_i \) was measured across the trajectory. Step 2) shows how that angle was thresholded at 130° to produce forward and backward moves. Step 3) shows the final converted observed sequence. C) Example of possible state transitions for the model.

When the HMM was applied to myosin XIa and VAMP72-labeled tracking data in protoplasts, it found 139 out of 307 (45%), and 126 out of 572 (22%),
trajectories with an active component, respectively (Figure 4.7). The HMM also estimated the most likely state transition probabilities for myosin XIa and VAMP72-labeled vesicles, Table 4.1 (see Methods). Importantly, these transition probabilities allowed us to determine lower limit estimates for the actin filament off-rates for myosin XIa and VAMP72-labeled vesicles. We found that both myosin XIa and VAMP72-labeled vesicles have approximately a 75% chance of remaining on a filament \( P(h_t = A|h_{t-1} = A) \) during the 50 ms exposure time. Consistent with this fast off-rate, we did not observe any active trajectories longer than 1.05 s. We also observed the actin-associated run lengths to have an end-to-end distance of 1.24 ± 0.06 and 1.30 ± 0.07 μm for myosin XIa and VAMP72-labeled vesicles, respectively (errors indicate standard error of the mean).

Table 4.1. Hidden Markov Model Protoplast Parameters. Transition and emission matrices for 3mEGFP-myosin XIa and 3mEGFP-VAMP72-labeled vesicles measured at the cell cortex. Emission matrices were held constant throughout parameter convergence. Here A and B indicate the hidden active and Brownian states, and F and R indicate the observed forward and reverse emissions.
To determine if VAMP72-labeled vesicle motion is dependent on myosin XIa, we examined the HMM results and the co-localized trajectories together, Figure 5.8. We found that of the 139 VAMP72-labeled vesicle active trajectories, only 28, 20%, of them were co-localized with a detectable myosin XIa. Since 80% percent of the VAMP72-labeled vesicles were able to move actively without a

Figure 4.7. Example hidden Markov model predicted trajectories in protoplasts A) Example VAMP72-labeled vesicle trajectories predicted by the HMM containing a sequence in the active state. B) Example myosin XIa trajectories predicted by the HMM containing a sequence in the active state. Red circles indicate the Brownian state and Green circles indicate the active state.

Xla we examined the HMM results and the co-localized trajectories together, Figure 5.8. We found that of the 139 VAMP72-labeled vesicle active trajectories...
myosin XIa, this suggests that there may be a population of VAMP72-labeled vesicles that move on microtubules or that some vesicles are propelled by formin-mediated actin polymerization (van Gisbergen et al., 2012; Furt et al., 2013). Since 19 out of the 126 (15 %) myosin XIa active trajectories were co-localized with a detectable VAMP72-labeled vesicle, myosin XIa may be able to move on actin without any cargo or myosin XIa might transport another cargo other than VAMP72-labeled vesicles.

**Figure 4.8.** Example co-localized protoplast trajectories. VAMP72-labeled vesicle trajectories (red dots) and myosin XIa trajectories (green dots) co-localized (yellow dots) exhibiting active motion (blue lines) predicted by the HMM.
4.3.4 Myosin XIa-associated Structures and VAMP72-labeled Vesicles Motility Depends on Actin in Caulonema

To demonstrate that the motility of both myosin XIa and VAMP72-labeled vesicles depends on actin in tip-growing cells not only in protoplasts, we imaged the apical caulonemal cells in the presence or absence of 25 μM latrunculin B, which completely depolymerizes the actin filaments (Vidali et al., 2009). When we applied our HMM to untreated caulonemal cells with fluorescently labeled myosin XIa and VAMP72 labeled-vesicle trajectories, we found in total 45 out of 5849 (0.77 %) and 11 all out of 2532 (0.43%), trajectories respectively, that were most likely to be moving along actin filaments, Figure 4.9. Upon treatment with latrunculin B, we found no trajectories that were likely to be moving along actin filaments out of the 4876 and 1342 myosin XIa and VAMP72-labeled vesicle tracks, respectively. This further demonstrates that myosin XIa and VAMP72 mobility is dependent on the presence of F-actin. It is important to note that the observed active trajectories in the caulonema are almost an order of magnitude lower than the number observed in protoplasts. Whether this is a product of poor tracking fidelity due to lower signal to noise ratios or physiologically relevant, is uncertain. The state transition probabilities for myosin XIa and VAMP72-labeled vesicles can be found in Table 4.2. We found that both myosin XIa and VAMP72-labeled vesicles have approximately a 75 % chance of remaining on a filament every 50 ms, which is similar to the protoplast results. Consistent with this fast off-rate, we did not observe any active trajectories longer than 0.55 s. We also observed the actin-associated run lengths to have an end-to-end distance of 0.85 ± 0.10 and 0.70 ±
0.08 \mu m for myosin XIa and VAMP72-labeled vesicles, respectively (error bars indicate standard error of the mean), which is also similar to the protoplast results. Consistent with our protoplast findings, this indicates that at the cortex of caulonemal cells, myosin XIa directed transport of vesicles along actin filaments is short lived and fast.

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Table 4.2. Hidden Markov Model Caulonema Parameters. Transition and emission matrices for 3mEGFP-myosin XIa and 3mEGFP-VAMP72-labeled vesicles measured at the cell cortex. Emission matrices were held constant throughout parameters convergence.
Figure 4.9. Example caulonema hidden Markov model predicted trajectories A) All the VAMP72-labeled vesicle trajectories predicted by the HMM to contain a sequence in the active state. B) Representative VAMP72-labeled vesicle trajectories in latrunculin B treated caulonema. C) All the myosin Xla trajectories predicted by the HMM to contain a sequence in the active state. D) Representative myosin Xla trajectories in latrunculin B treated caulonema. Red circles indicate the Brownian state and green circles indicate the active state.
As an alternative to the HMM, we applied another approach based on trajectory filtering. We focused on punctate structures that display a directed movement for at least 12 consecutive frames (600 ms) and show a displacement higher than 1.2 \( \mu m \). Examples of such persistent linear trajectories recorded for myosin XIa and VAMP72 are shown in Figures 4.10A and 4.11A, respectively. Over a period of 100 s, we were able to detect 20 and 13 persistent linear trajectories per average imaged area per cell (40 \( \mu m^2 \)) for myosin XIa and VAMP72, respectively (Figures 4.4B and 4.5B). However, after treatment with latrunculin B, we found only 4 and 1 persistent linear trajectories per average imaged area per cell (40 \( \mu m^2 \)) for myosin XIa and VAMP72, respectively (Figures 4.10B and 4.11B). Such a decrease in the number of persistent linear trajectories in the absence of actin filaments further supports the hypothesis that myosin XIa- and VAMP72-labeled endomembrane vesicles move on actin filaments in apical caulonemal cells.
Figure 4.10. Myosin XIa directed motion at the cell cortex depends on actin. A) Two representative examples of persistent linear trajectories of 3mEGFP-Myosin XIa at the cortex of apical caulonema cells near the apex. Images were acquired using VAEM at 50 ms intervals for the complete time series. Selected images of each trajectory are shown with the time from the series indicated at the top. Persistent linear trajectories are shown in the last image of each series. Scale bar is 1 μm. B) Quantification of the number of persistent linear trajectories detected in control and latrunculin B treated apical caulonema cells (n=6 cells for both conditions). Error bars correspond to the standard errors of the mean.

*p<0.01
4.3.5 Apical Myosin Xla Recovers Before VAMP72 at the Cell Apex in an Actin-Dependent Manner

Since myosin Xla at the apex of tip-growing caulonemal cells cannot be imaged with variable epifluorescence microscopy, we conducted Fluorescence Recovery
After Photobleaching (FRAP) experiments (McNally, 2008; Loren et al., 2015) to probe the dynamics of myosin XIa at the growing cell tip and shank (Figure 5.12A). Compared to the shank, myosin XIa at the tip exhibited an increased rate of fluorescence recovery. This suggests that F-actin, at the cell tip, increases the flow of myosin XIa when compared to the shank. This is in contrast to a mechanism by which F-actin statically captures myosin XIa for increased accumulation at the cell tip, which should result in a slower recovery.

To better understand how F-actin increases the flow of myosin XIa at the cell tip, we analyzed its fluorescence recovery spatially. At early times during fluorescence recovery (1.13 s) we found that myosin XIa could be first seen recovering at the cell apex before VAMP72-labeled vesicles (Figure 4.12B). Given that the bleach region is 4 μm in diameter, and the observed velocity of myosin XIa on actin filaments is roughly 1.5 μm/s, it is reasonable that actin-mediated myosin

**Figure 4.12.** Apical Myosin XIa dynamics during polarized cell growth. A) Fluorescence recovery of 3mEGFP-myosin XIa at the cell shank (green circles) and the cell tip (black circles). n=7 and 10 for the shank and tip, respectively. (Error bars represent standard deviations). B) Cropped and frame averaged photobleaching ROI at the cell tip for cells expressing 3mEGFP-myosin XIa (left) and 3mCherry-VAMP72 (right). White circle and arrows indicate how the perimeter of the ROI was measured during the recovery. C) Intensity profile of 3mEGFP-myosin XIa (left) and 3mCherry-VAMP72 (right) along the perimeter of the ROI 1.13 s following photobleaching.
XIa transport is responsible for the observed fluorescence recovery. To quantify the directionality of this recovery, we measured the fluorescence of myosin XIa along the perimeter of the photobleaching region (Figure 4.12C).

To determine if this recovery was actin dependent, we treated growing cells with latrunculin B. Treatment reduced the flow of myosin XIa at the cell tip (Figure 4.13A) and changed the direction of myosin XIa recovery (Figure 4.13B). This indicates that myosin XIa exhibits an actin-dependent apical recovery. However, if this recovery was only the result of vesicle-mediated myosin transport, myosin should recover apically as quickly as VAMP72-labeled vesicles. Since this is not the case (Figure 4.13B), myosin XIa must exhibit another mode of recovery. Although myosin XIa diffusion contributes to fluorescence recovery on these time scales, it alone cannot produce an apical localization. This leaves three potential modes of recovery that could result in apical myosin XIa recovery that precedes the recovery of VAMP72-labeled vesicles; first is possible that unloaded myosin XIa or myosin XIa transporting a different cargo moves processively along actin filaments and contributes to recovery before vesicle loaded myosin XIa. Second, myosin XIa might be binding and unbinding rapidly with actin filaments without active transport. Lastly, myosin XIa might bind and unbind with photobleached VAMP72-labeled vesicles at the cell apex.
4.3.6 Quantitative Exploration of Myosin XIa Vesicle Binding with FRAP

To further assess how myosin XIa associates with VAMP72-labeled vesicles, we wanted to determine if our previously measured binding constants are consistent with our fluorescence recovery data. To characterize the kinetics of this reaction with FRAP, we took advantage of the fact that myosin XIa should diffuse more quickly than myosin XIa attached to a vesicle. To probe this dynamic information, we conducted latrunculin B FRAP experiments (Figure 4.13A). By removing the actin cytoskeleton, we could ensure that myosin XIa fluorescence recovery was a result of myosin XIa either bound or unbound to vesicles. Because the fluorescence recovery of 3mEGFP labeled myosin XIa is made up of both unbound and bound myosin XIa, the observed FRAP should then exhibit an intermediate

![Figure 4.13](image-url)
rate of recovery between the myosin XIa diffusion coefficient and the vesicle diffusion coefficient. The more myosin XIa bound to vesicles, the more the recovery should look like a vesicle recovery. Based on this recovery it is possible to determine information regarding myosin XIa and vesicle binding interactions if the diffusion coefficient of myosin XIa and vesicles are known, and are significantly different. Since myosin XIa diffusion cannot be measured with FRAP, due to the potential of vesicle binding, we estimated a potential range of diffusion coefficients based on its known size. As an upper limit estimate, we assume that the full

**Figure 4.14.** Theoretical myosin XIa diffusion based FRAP. Fluorescence recovery of 3mEGFP-myosin XIa in latrunculin B treated cells at the shank (green circles) compared to theoretical myosin XIa diffusion where $D = 1.3$ (gray), 4.3 (black), or 0.29 μm$^2$/s (blue).

myosin XIa dimer complete with 3mEGFP and calmodulins has a molecular weight of 746kD. Based on previous measurements of unconjugated 3mEGFP, and that hydrodynamic radius of a spherical, globular protein should increase with the inverse cube a protein’s molecular weight, we estimate that the upper limit myosin XIa diffusion coefficient is 4.3 μm$^2$/s (McNally, 2008). Based on electron
micrographs of tobacco myosin XIa, we can estimate that the hydrodynamic radius of myosin XIa to be 0.025 μm (Tominaga et al., 2003). With this hydrodynamic radius, the lower limit for myosin XIa diffusion becomes 1.3 μm²/s.

Applying our lower limit estimate for myosin XI diffusion to the analytical model from Soumpasis for diffusive FRAP, yields a recovery curve that is indistinguishable to the moss recovery data at the shank (Soumpasis, 1983), (Figure 4.14). This suggests that our lower limit estimate is too slow because we observe some binding with particle tracking, but more importantly indicates that FRAP may not be sensitive enough to measure any binding interactions between myosin XIa and vesicles. However, we can calculate an upper limit estimate of the myosin XIa bound to vesicles, because our upper limit estimate of the myosin XIa diffusion is significantly faster than our experimental recovery curve (Figure 4.14). Thus using reaction-diffusion modeling of FRAP, we can establish an upper limit estimate of the percent of myosin XIa on vesicles.
To simplify FRAP of reversible binding of myosin XIa and vesicles we assumed that the number of myosin XIa binding sites on a vesicle, $S(x,y,t)$, are at equilibrium, $S(x,y,t)=S_{eq}$ (Sprague and McNally, 2005; Kang and Kenworthy, 2008; Kang et al., 2010). It then follows that we can write the following expression for the effective on-rate $k_{on}^*=S_{eq}k_{on}$ (Sprague and McNally, 2005; Kang and Kenworthy, 2008; Kang et al., 2010). Based on these assumptions, we can now model the process as a two-species reaction-diffusion system described by Eqs. 4.5 and 4.6 (Kang et al., 2010, Kang & Kenworthy, 2008), i.e.,

\[
\frac{\partial U(x,y,t)}{\partial t} = \nabla \cdot \left( D_U \cdot \nabla U(x,y,t) \right) + k_{off} B(x,y,t) - k_{on}^* U(x,y,t) \quad (Eq. 4.5)
\]

\[
\frac{\partial B(x,y,t)}{\partial t} = \nabla \cdot \left( D_B \cdot \nabla U(x,y,t) \right) - k_{off} B(x,y,t) + k_{on}^* U(x,y,t).
\quad (Eq. 4.6)
\]
Here, $U(x,y,t)$ and $B(x,y,t)$ are the concentrations of unbound and bound myosin XIa, $D_U$ and $D_B$ are the estimated diffusion coefficients for unbound myosin XIa and VAMP72-labeled vesicles, $\nabla$ is the gradient operator, and $k_{\text{off}}$ is the myosin XIa vesicle off-rate.

Although there are several analytical solutions to Eqs. 4.5 and 4.6 (Sprague and McNally, 2005; Kang and Kenworthy, 2008; Kang et al., 2010), previous evidence suggests (Section 2) that these models can be sensitive to cell boundaries imposed by the cell membrane (Bibeau et al., 2017). For that reason, we used the finite element modeling software Comsol (Comsol Inc, Stockholm, Sweden) to simulate FRAP in the presence of myosin vesicle binding (Figure 4.15A) (see Methods). A parameter sweep across potential reaction on and off-rates (Figure 4.15B) demonstrates that we only have the experimental sensitivity to detect the effective dissociation constant $K_d^* = k_{\text{off}}/k_{\text{on}}^*$, and not the individual rate constants. Using the Matlab (Mathworks, Natick, MA) liveLink Comsol Multiphysics module, we were able to iteratively find a dissociation constant that matched experimental myosin XIa fluorescence recoveries, $K_d^* = 0.33$ at both the tip and the shank. A similar result was found by applying the two-dimensional infinite-boundary reaction diffusion model from Kang et al. to the fluorescence recovery at the cell shank, $K_d^* = 0.31$ (Figure 4.16) (Kang et al., 2010). Using the Digital Confocal Microscopy Suite developed by (Kingsley et al., 2018), we were able to match the experimental recovery curves given $K_d^* = 0.31$ (Figure 4.16).
This upper limit $K_d^*=0.31$ is significantly smaller than what we observed in protoplasts $K_d^*\sim22$. This upper limit effective dissociation constant $K_d^*$ would yield, an upper limit true dissociation constant of $K_d = 0.47-0.717 \mu M$ and an average of 1.45 myosin Xla motors per VAMP72-labeled vesicle. Despite this difference, our observed $K_d^*$ in protoplast is still consistent with our FRAP data because it is well within the lower limit estimate which indicates that no binding is a possibility. If we use the protoplast effective dissociation constant as a known parameter in the model from Kang et al. and use the myosin XI diffusion coefficient as a fitting parameter, we get a myosin Xla diffusion coefficient of $1.47 \mu m^2/s$, which is within our estimated range of 4.3 and 1.3 $\mu m^2/s$.

**Figure 4.16.** Reaction-diffusion models estimate an upper limit $K_d^*$. Fluorescence recovery of 3mEGFP-myosin Xla in latrunculin B treated cells at the shank (green circles) and corresponding fit with the two-dimensional infinite-boundary reaction-diffusion model from (Kang et al., 2010) (red dotted lines). Blue line indicates the fluorescence recovery from the Developed Digital Confocal Microscopy Suite with an input $K_d^*=0.31$. 

![Figure 4.16](image-url)
4.4 Discussion

In this work, we have shown that myosin XIa and VAMP72-labeled vesicles co-localize, and exhibit actin dependent mobility that was observed as short persistent trajectories. Using particle-tracking statistics, we found that myosin XIa exhibits a low-affinity interaction with VAMP72-labeled vesicles. Taken together, this work supports a hypothesis in which myosin XIa transiently interacts with its binding constituents to flexibly coordinate vesicle motion and actin dynamics.

To demonstrate that myosin XIa associates with vesicles, we performed Variable Angle Epifluorescence Microscopy (VAEM) on protoplasts expressing fluorescently labeled myosin XIa and VAMP72-labeled vesicles. With the particle tracking plugin, TrackMate, we found that myosin XIa co-localizes with VAMP72-labeled vesicles for periods that would support an off-rate of $2 \times 10^{-1}$. Because the proteins can move out of the evanescent field, this is an upper limit approximation of the off-rate. The degree to which this is an overestimate is related to the diffusion coefficient of a vesicle, and the penetration depth of the evanescent field.

We also observed for the first time, to the best of our knowledge, several instances in which fluorescently labeled myosin XIa associated and moved along life-act decorated actin filaments. Using two color imaging of myosin XIa and VAMP-labeled vesicles in conjunction with a Hidden Markov Model (HMM), we examined how co-localization influenced their respective dynamics. We found that active motion of myosin XIa and VAMP72-labeled vesicles could happen with or without an observed co-localization. This indicates that myosin XIa either transports cargo other than VAMP72-labeled vesicles or that myosin XIa can move unloaded on filamentous actin. VAMP72-labeled vesicles may also move on
microtubules or through the formin-mediated actin propulsion system previously suggested (Binder and Holzhutter, 2012).

To further explore how the dynamics of myosin XIa and VAMP72-labeled vesicles depends on actin in tip-growing cells, we performed VAEM at the cortex of caulonema cells expressing fluorescently labeled myosin XIa or VAMP72-labeled vesicles. We found that myosin XIa and VAMP72-labeled vesicles traveled along actin filaments with run lengths around 0.8 μm, for periods lasting no longer than 0.5 s. To further demonstrate the actin dependence of these trajectories, we treated caulonemal cells with latrunculin B, and found no actively transported trajectories via the HMM. This suggests that all the observed myosin XIa active motion happens along filamentous actin. However, we observed VAMP72-labeled vesicles active motion in protoplasts without any observed localization to a myosin XI. Although we observed no localization to myosin XI, it is possible that this VAMP72-labeled active motion is myosin XI dependent and we don’t have the resolution to detect it.

To better understand how myosin XIa and VAMP72-labeled vesicles interact with the actin cytoskeleton at the tip of the cell during polarized growth we performed Fluorescence Recovery After Photobleaching experiments (FRAP). Here we found that myosin XIa and VAMP72-labeled vesicles exhibited an actin-dependent fluorescence recovery. During fluorescence recovery, we found that both myosin XIa and VAMP72-labeled vesicles restored the apical localization at the cell tip in under 2 s. Considering that we used a bleach radius of 2 μm, and that the myosin XIa and VAMP72-labeled vesicles exhibit transport velocities
around 2 μm/s, these recovery rates are consistent with active transport. However, we did find that myosin XIa was able to achieve apical localization before VAMP72-labeled vesicles. This indicates that myosin XIa displays another tip-localized recovery mode independent from, diffusion and VAMP72 vesicle transport. This recovery mode maybe unloaded myosin XIa on actin or myosin XIa exchanging off of bleached vesicles at the tip and is left for future work. Nevertheless, the apical recovery rates seen here, and the myosin XIa movement without VAMP72-labeled vesicles, are consistent with the active transport found by our HMM at the cell cortex.

To determine the affinity between myosin XIa and vesicles in tip-growing cells, we performed FRAP on latrunculin B treated caulonemal cells. Using the finite element modeling of reaction-diffusion, we determined an upper limit for the effective dissociation constant $K_d' = 0.31$. At the lower limit, the reaction-diffusion models predict no binding between myosin XIa and vesicles. This measurement can be better constrained with the true diffusion coefficient for myosin XI.

Based on the particle tracking evidence and the bulk FRAP measurements we conclude that the effective dissociation constant of myosin XIa must fall between $K_d' = 0.31-22$. Since *P. patens* does not exhibit large organelle cytoplasmic streaming (Furt et al., 2012), it is not surprising that myosin XIa only displays short-lived transport of vesicles. Additionally, the myosin XIa actin-transport speeds found here are slow relative to other orthologs (Tominaga et al., 2013) and maybe a product of the slow polarized growth found in moss. Importantly, the weak interactions and short run lengths we observed are
consistent with a mechanism by which myosin XIa can actively remodel and regulate the oscillating apical F-actin, during polarized growth, while still promoting vesicle focusing for exocytosis see Figure 4.17. Without the flexibility of these weak transient interactions, the apical actin spot may not be as dynamic or easily regulated. Thus, we believe that the affinities observed here are likely to be essential constants that give important insight into polarized growth.

**Figure 4.17.** Transient interactions between myosin XIa, VAMP72-labeled vesicles, and F-actin give rise to system flexibility in polarized cell growth. Here myosin XIa and vesicles transiently interact with each other and the actin cytoskeleton to give rise to short lived persistent motion directed toward the secretion zone at the cell apex. These transient interactions allow or the dynamic actin oscillations observed during tip growth.

### 4.5 Methods

#### 4.5.1 Constructs and Cell Lines

All cell lines described in this study were derived from the moss *Physcomitrella patens* (Hedw.) Bruch and Schimp., Gransden strain (Wild type)
and are listed in Table 4.1. Two independent 3mEGFP-VAMP72 lines were obtained by transforming the Gransden strain with pTH-Ubi-3mEGFP-VAMP72, and by selecting for stable lines. The construct was obtained by a two element LR Gateway reaction of entry clones pENT-L1-3mEGFP-R5 and pENT-L5-VAMP72-L2 (Furt et al., 2013) and the destination vector pTH-Ubi-Gateway (Vidali et al., 2007). Three independent lines 3mCherry-RabA plus 3mEGFP-MyosinXia were obtained by transforming the existing 3mEGFP-MyosinXia line (Vidali et al., 2010) with pTH-Ubi-3mCherry-RabA and by selecting for stable lines. The construct was obtained by a two element LR Gateway reaction of entry clones pENT-L1-3mCherry-R5 and pENT-L5-RabA-L2, and the destination vector pTH-Ubi-Gateway (Vidali et al., 2007). The 3mCherry-VAMP72 plus 3mEGFP-MyosinXia and the Lifeact-mCherry plus 3mEGFP-MyosinXia lines were previously described (Furt et al., 2013).

4.5.2 Culture Conditions

*Physcomitrella* cell lines were propagated using standard methods as previously described (Vidali et al., 2007). Isolation of protoplasts and DNA transformation were done according to Liu and Vidali (Liu and Vidali, 2011).

For the VAEM imaging (Konopka and Bednarek, 2008), moss cell lines were grown on PpNO$_3$ medium (Vidali et al., 2007) for 7 days. One week-old protonemal tissue was either directly mounted on glass microscope slide for imaging or used to prepare protoplasts as previously described with some modifications (Liu and Vidali, 2011). After counting, protoplasts were resuspended in 5 ml of 8 % (w/v) mannitol and cultured in the growth chamber for one day. For imaging, moss
protonemal tissue or protoplasts were deposited on a 1 % (w/v) agar pad in PpNO₃
medium on a glass microscope slide. 10 μL of liquid PpNO₃ medium was applied
before covering with a glass 0.25 mm thick coverslip and sealing with VALAP
(1:1:1, VAseline, LAnolin, Paraffin). For latrunculin B treatments, 10 μL of 25 μM
latrunculin B resuspended in PpNO₃ medium was added to the preparation instead
of the PpNO₃ medium alone. Apical caulonemal cells were imaged exactly 10 min
after the treatment.

For FRAP, moss cell lines were grown and cultured as described in (Bibeau
et al 2017 section 3.5.2).

4.5.3 VAEM Microscopy

Protoplasts were mounted on an inverted microscope (model Ti-E; Nikon
Instruments) and imaged with an Apo TIRF, 60 x, NA 1.49, oil immersion objective
(Nikon Instruments). Apica caulonemal cells from protonemal tissue were imaged
with an Apo TIRF, 100x, NA 1.49, oil immersion objective (Nikon Instruments). To
increase magnification, the 1.5x optivar was used to collect all images. The 488
and 561 nm laser lines were used to excite mEGFP and mCherry, respectively. To
provide the maximum signal-to-noise ratio, the laser illumination angle was finely
tuned for each sample. Signals were simultaneously acquired for both channel
using a beam splitter and a 512x512 electron-multiplying CCD camera iXON3
(Andor Technology).

4.5.4 Numbers and Brightness Analysis

Brightness and numbers analysis was conducted as outlined in (Bibeau et al 2017
section 3.5.6), with the exception that the imaging laser power was set to 15%.
4.5.5 Image Processing and Tracking

Images collected by VAEM, were first processed in ImageJ using a background subtraction (rolling ball radius was tuned to 5 for myosinXI and VAMP72), a FFT bandpass filter (a diameter comprised between 2 and 20 pixels was used) and an enhance contrast (enhancement with no pixel saturation was normalized to all slices of each time series) tool. Detection and tracking of the cortical punctate structures of myosin Xla and VAMP72 were performed using the TrackMate plugin in FIJI. We manually tracked several punctate structures to determine the best settings. Briefly, the DoG Detector tool was chosen because it is optimal for small spot sizes and it allows to differentiate between two spots that are close to each other. The diameter of the structures was no greater than 0.6 μm and the threshold was kept at 1000 for all molecules analyzed. For tracking, we used the simple LAP tracker tool with the following parameters: linking maximum distance of 0.5 μm; gap-closing maximum distance of 0.7 μm; gap-closing maximum frame gap of 0. Two other filters were applied to track the punctate structures that move in a linear persistent manner: a number spots per track greater than 12 (equivalent to 600 ms) and a displacement greater than 1.2 μm. Each trajectory recorded was verified manually using an in-lab developed Matlab routine. To track the punctate structures that stay confined to an area, we adjusted the filters as follow: a number of spots per track greater than 90 (equivalent to 4.5 s) and a displacement no greater than 0.5 μm. The number of trajectories was normalized by average imaged area (40 μm²) for each cell. The instantaneous velocities of each tracked punctate structure were calculated using the coordinates obtained by the simple LAP tracker tool.
4.5.6 Co-localization Frequency

To automate the identification of co-localized fluorescently labeled myosin XIa and VAMP72-labeled vesicles we used an approach similar to (Deschout et al., 2013). With a custom Matlab (Mathworks Natick, MA) routine we compared the myosin XIa and VAMP72-labeled trajectories. An example analysis can be found in Figure 4.18. In order for two trajectories to be considered co-localized they first needed to have a temporal overlap and be within a specified contact radius of 0.5 μm.

Then a sliding correlation window was used to determine the correlations between the two channels in the x and y directions, respectively. If the correlation within the window was greater than 0.7 the movement was classified as correlated for that direction. Window correlations were found using the Matlab function, corr. If the trajectories exhibited correlated motion in both the x and y directions the time point within the selected window was classified as co-localized. Here we used a minimum correlation threshold of 0.7 and a maximum contact radius of 0.5 μm to account for tracking and alignment error.

To demonstrate that the observed co-localized trajectories were not a result of random Brownian motion, we randomly shuffled the existing trajectories in time and space with a custom written Matlab script. The new trajectories were then analyzed for co-localization as previously mentioned.
To classify the state of a particle for a given point in a trajectory, we employed a Hidden Markov Model (HMM) similar to the one used in (Roding et al., 2014). All trajectories were preprocessed such that they were converted from spatial...
information into either reverse, $R$, or forward, $F$, moves. Specifically, we measured the angle between three consecutive points along the trajectory, namely, $t_{i-1}$, $t_i$, and $t_{i+1}$. This angle $\theta_i$ was then classified as a forward move if the angle was greater than $130^\circ$ or classified as a reverse move if less than $130^\circ$. $130^\circ$ was chosen because it allowed for the detection of shorter active trajectories while minimally effecting the most likely transition probabilities (Figure 4.19). Larger angles placed too stringent of a requirement on forward moves and began to influence the transition matrix (Figure 4.19). These sequences of forward and backward moves were then applied to an HMM with two hidden states, namely, an active state, $A$, and a Brownian state, $B$. Here, we represent each hidden sequence as $H=h_1,h_2,\ldots,h_T$ and each observed sequence as $Q=q_1,q_2,\ldots,q_T$. In the active state, $A$, we assumed that myosin XIa and VAMP72-labeled vesicles always move forward, while in the Brownian state, $B$, we assumed an equal probability distribution over all angles, $\theta$. Thus the emission probabilities in the Brownian state are only a function of the angle threshold, where the probability of moving forward

![Figure 4.19. Transition matrix sensitivity to the chosen angle threshold. Hidden Markov Model myosin XIa (A) and VAMP72-labeled vesicle (B) transition probabilities in protoplasts, as a function of the angle threshold. Red lines indicate the Brownian to Brownian transition probability, $P(h_t = B \mid h_{t-1} = B)$. Green lines indicate active to active transition probability, $P(h_t = A \mid h_{t-1} = A)$.](image-url)
given the Brownian state and the probability of moving in the reverse direction
given the Brownian state are, \( P(q_t = F \mid h_t = B) = (180-130)/(180) \) and \( P(q_t = R \mid h_t = B) = 1 - P(q_t = F \mid h_t = B) \), respectively. This leaves the state transition probabilities as the only open parameters in our model. The state transitions probabilities are the chances that protein of interest will change its state on each successive portion of the sequence. The four possible state transitions are as follows, active to active, active to Brownian, Brownian to Brownian, and Brownian to active which can be written formally as \( P(h_t = A \mid h_{t-1} = A) \), \( P(h_t = A \mid h_{t-1} = B) \), \( P(h_t = B \mid h_{t-1} = B) \), and \( P(h_t = B \mid h_{t-1} = A) \), respectively.

To find the most likely state transitions for each experimental condition, we used the forward and reverse trajectories as inputs to a modified version of the Matlab function \textit{hmmtrain} for known emission matrices. \textit{hmmtrain} uses the Baum Welch algorithm, which recursively finds the transition and emission matrices that maximize the likelihood of the observed sequence. We modified this algorithm

**Figure 4.20.** Transition matrix optimization. Log-likelihood of all the observed sequences of VAMP72-labeled vesicles (A) and myosin Xla (B) in protoplasts for an array of possible transition matrices. x and y-axes represent \( P(h_t = A \mid h_{t-1} = B) \) and \( P(h_t = B \mid h_{t-1} = B) \), respectively. Blue to yellow color map indicates log-likelihood where blue indicates less likely observed sequences and yellow indicates more likely sequences. A clear global maximum can be found in both (A) and (B).
such that the emission matrix did not change. This modified function reliably found the transition matrix that maximized the likelihood of the observed sequence $Q$, regardless of the initial guess for the transition matrix (Figure 5.20). With the most likely state transitions and the converted sequences, we then used the Matlab function `hmmviterbi` to determine the most likely state of a specific particle at a specific time.

4.5.8 Experimental FRAP Processing
Experimental FRAP was processed as previously described in, (Bibeau 2017 sections 3 and 4). Briefly, FRAP confocal images were saved as tiff stacks and converted into intensity traces by averaging the mean fluorescence intensity within the 4 μm in diameter region of interest, ROI, using a custom written imageJ macro. All replicate experiments were then averaged and normalized by acquisition photobleaching controls with Matlab. To quantify the spatial fluorescence recovery FRAP tiff stacks had the respective ROIs cropped and averaged using a custom written imageJ macro. Then the fluorescence intensities of the perimeters of the cropped ROIs were extracted, limited volume corrected, and plotted in Matlab.

4.5.9 Modeling Reaction Diffusion FRAP
To solve the reaction diffusion equations Eqs. 4.5 and 4.6 in COMSOL (Comsol Inc, Stockholm, Sweden) we used no flux boundary conditions inside a two-dimensional approximation of the moss geometry. We also used initial conditions in which $U(x,t,0) = \text{total}/(1+K_d^\ast)$ and $B(x,y,0) = \text{total}-U(x,y,0)$ for everywhere outside the 4 μm diameter circular bleach region, $R$. Here the effective equilibrium constant is the ratio between the effective on-rate and off-rate, $K_d^\ast = k_{on}^\ast/k_{off}^\ast$, and
total is the arbitrary combined concentration of the unbound and bound myosin XIa. This concentration is arbitrary, because we observe experimentally the normalized fluorescence intensity within the region of interest, i.e.,

\[
int(t) = \iint_{R^2} \frac{(U(x,y,t)+B(x,y,t))}{(U(x,y,0)+B(x,y,0))} \, dx \, dy.
\]

Eq. 4.7

Inside the bleach region the initial concentrations for \( U(x,y,0) \) and \( B(x,y,0) \) were multiplied by the bleach depth parameter, \( BD \), to simulate an instantaneous ideal bleach.

To solve these equations for arbitrary \( k_{on}^* \) and \( k_{off} \) we used the finite element modeling software Comsol Multiphysics (Comsol Inc, Stockholm, Sweden). Simulations were created using the “two-dimensional transport of dilute species” interface with two species, a “time-dependent solution”, and a “normal physics controlled mesh”. We then performed a parameter sweep across potential values of \( k_{on}^* \) and \( k_{off} \) using the Matlab liveLink Comsol Multiphysics module and calculated the sum of squares differences between the simulated recoveries and those measured experimentally. Based on these results we concluded that we could only determine the effective dissociation constant, \( K_d^* = k_{on}^* / k_{off} \). To converge on a best-fit \( K_d^* \) we imposed gradient descent on the following cost function,

\[
J(K_d^*) = \sum \left[ int_{\text{tip}}^{\text{sim}}(t)K_d - int_{\text{tip}}^{\text{exp}}(t) \right]^2 + \sum \left[ int_{\text{shank}}^{\text{sim}}(t)K_d - int_{\text{shank}}^{\text{exp}}(t) \right]^2,
\]

(Eq. 4.8)
using a custom written script for the Matlab liveLink Comsol Multiphysics module. Here the superscripts \( sim \) and \( exp \) indicate simulated and experimental FRAP recoveries, the subscripts indicate the position of the photo-bleach, and \( J(K_d) \) is the dissociation constant dependent cost function. Here we used the numerical forward approximation of a derivative to determine the local gradients during convergence.
5 Future Directions

5.1 Further Characterization of Myosin XI and Vesicle Binding

Due to the poor resolution provided by FRAP, the characterization of myosin XIa and vesicle binding is heavily dependent on tracking fidelity. The tracking fidelity of Trackmate must be validated and or optimized. Thus a dual color VAEM simulation was written, it should be used to check the accuracy of Trackmate and downstream analysis such as the co-localization algorithm and the hidden Markov model. To improve accuracy of VAEM simulations, the experimental evanescent field and PSFs should be measured. The necessary codes have already been written.

More experimental VAEM images of myosin XIa and VAMP72-labeled cells in caulonema would also help complete the understanding of this interaction in tip-growing cells. Brightness and Numbers analysis should be conducted on VAMP72-labeled vesicles and myosin XI in protoplasts as opposed to the current caulonema measurements.

Additionally, cross raster correlation image spectroscopy (crossRICS) can also be used as a technique to validate myosin XIa and VAMP72-labeled vesicle binding. This should ideally be conducted with photon counting detectors for each imaging channel to reduce noise. The analysis script has already been written. All analysis can be validated with the existing version of DCMS with reaction kinetics.
5.2 Characterization of Formin Vesicle Binding

In the vesicle organizing center model proposed by (Furt et al., 2013), one of the key components is a formin bound to secretory vesicles. This formin is thought to polymerize on vesicles to yield cell polarization. To test this component of the model, co-localization and the hidden Markov model can be applied to caulonema and protoplast VEAM tracking data of fluorescantly labeled formin and VAMP-72-labeled vesicles. Currently, preliminary tracking data suggests a significant interaction. The hidden Markov model also suggests that a very large portion of formin exhibit active trajectories, possibly via active transport or actin polymerization events. With more data and the established analysis tools, this work could shed light on the accuracy of the vesicle organizing center model.

5.3 How Cell Shape Influences Analysis of Reaction-Diffusion FRAP

In section 3, the effects of cell shape on FRAP were outlined. A natural next step in this analysis would be to explore the effects cell shape has on reaction-diffusion FRAP. These effects were taken into account in section 4, but their exact influence on recovery was not discussed for the sake of brevity. Currently, there are two prominent infinite boundary models for reaction-diffusion FRAP. In the model proposed by Sprague et al. (Sprague et al., 2004), there is one fluorescent diffusing species and a static receptor that the diffusing species reversibly binds to. The second model for reaction-diffusion FRAP is one in which a protein with a fast diffusion coefficient binds to a complex with a slower diffusion coefficient (Kang et al., 2010). Both models have numerical fitting functions that have been written.
These models can be fit to DCMS data with reaction-diffusion using the various cell shapes outlined in (Kingsley et al., 2017) (including the membrane examples). Bleaching can be conducted at the cell center and edge. Numerical model fitting can then be used to test for boundary effects. To reduce the number of parameters in the fitting routines, it is advisable that all diffusion coefficients should be treated as known user inputs. Simulations should be conducted in which the fast species, species A, always has a diffusion coefficient of 10 μm²/s. The slow species, species B, should have a range of diffusion coefficients similar to the following, motionless, 0.1, 1, and 5 μm²/s. The simulations should also exhibit a range of binding conditions, from weak to strong binding. In this way, the performance of each model can be examined by comparing the predicted on and off rates, and the predicted effective dissociation constant. It is important to predict the dissociation constant because fitting may never have the sensitivity to resolve the individual on and off rates.

Each of the numerical two models above (Sprague and McNally, 2005; Kang et al., 2010) have special cases in which their forms can be simplified. These special cases should help interpret the performance of the models during the analysis. In Sprague et al., there are three special cases namely pure-diffusion dominant, effective diffusion, and reaction dominant. In the pure-diffusion dominant case binding is minimal and the diffusion of species A is all that influences the recovery. In this limit, the results should be identical to section 2. The second case is effective diffusion. In this case, during recovery, the reaction happens much faster than diffusion such that the bleach spot reaches a local
equilibrium almost instantly. Here a simple diffusion model can be used to fit the recovery data to obtain an effective diffusion coefficient, $D_{eff}$, where $D_{eff} = D/(1 + 1/K_d^r)$. This simple case yields an analytical expectation for the effect boundaries should have on measured reaction rates when coupled with the analysis previously performed in section 2.3.1. Specifically, the measured effective diffusion coefficient will be slowed by a factor of 4 in one-dimensional FRAP, and consequently, the incorrect effective dissociation constant predicted by the models will be $K_{d^*} = (D_{eff}/4) - 1$. The last case is the reaction dominant case, in which diffusion is much faster than the reaction. For that reason, the recovery is a function of only the reaction off-rate. Since there is no spatial component in this case, boundary effects should not influence the recovery.

The special case for the second model in which there are two diffusing species is the effective diffusion case. In this case, the reaction rates are much faster than diffusion. In this case, a simple diffusion model can be fit to the recovery data to obtain an effective diffusion coefficient, where $K_{d^*} = (D_{eff} - D_B)/(D_A - D_{eff})$ (Kang et al., 2010). Similar to the first model, this can also be coupled with the analysis performed in section 2.3.1 to determine analytically how cell shape can influence measured $K_{d^*}$. It is important to note that there is no reaction dominant case; this is because the recovery will always depend on diffusion because there are no static species. In its entirety, this study will help demonstrate the sensitivity reaction diffusion FRAP has at boundaries and help advance the field of fluorescence dynamics.
6 Conclusions

Substantial modeling and experimental efforts have shaped the current understanding of polarized cell growth. However, up until this point there has been little to no mechanistic understanding of the role of the actin cytoskeleton in polarized cell growth. For this reason, the work in this manuscript has been focused on providing a more mechanistic understanding of cell growth. To this aim, we used modeling in conjunction with in vivo experiments to measure important parameters in polarized cell growth. To permit the measurement of diffusion in the confined geometry of tip-growing cells, we first developed a particle based diffusion simulation that takes into account cell shape. This simulation can be applied other cell shapes and will help advance in vivo fluorescence dynamics measurements. With this simulation, we then measured the diffusion coefficient of vesicles inside tip-growing cells. With this measurement, along with an array of other physical parameters, we were able to build a diffusion-based growth model. This model demonstrates that the active transport of vesicles is required to support the vesicle fusion events necessary to drive cell growth. With VAEM of fluorescently labeled myosin X1a and vesicles and a hidden Markov model, we demonstrated that myosin X1a, VAMP72-labeled vesicles, and filamentous actin all exhibit transient interactions in vivo. This is the first time these interactions have been characterized in plants in vivo. Our measurements of the kinetics of these interactions will provide important parameters in future modeling efforts. These efforts can potentially incorporate the specific components of the cytoskeleton and the more established mechanics of turgor pressure induced cell wall expansion.
Taken together, this work has shown why actin is required in polarized cell growth and lays the foundation for future modeling studies involving the cytoskeleton, which will give rise to a more mechanistic understanding of the role of the cytoskeleton in polarized cell growth.
7 References

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