Magnitude and Direction of Vesicle Dynamics in Growing Pollen Tubes Using Spatiotemporal Image Correlation Spectroscopy and Fluorescence Recovery after Photobleaching¹[W][OA]

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The delivery of cell wall material and membrane to growing plant cell surfaces requires the spatial and temporal coordination of secretory vesicle trafficking. Given the small size of vesicles, their dynamics is difficult to quantify. To quantitatively analyze vesicle dynamics in growing pollen tubes labeled with the styryl dye FM1-43, we applied spatiotemporal correlation spectroscopy on time-lapse series obtained with high-speed confocal laser scanning microscopy recordings. The resulting vector maps revealed that vesicles migrate toward the apex in the cell cortex and that they accumulate in an annulus-shaped region adjacent to the extreme tip and then turn back to flow rearward in the center of the tube. Fluorescence recovery after photobleaching confirmed vesicle accumulation in the shoulder of the apex, and it revealed that the extreme apex never recovers full fluorescence intensity. This is consistent with endocytotic activity occurring in this region. Fluorescence recovery after photobleaching analysis also allowed us to measure the turnover rate of the apical vesicle population, which was significantly more rapid than the theoretical rate computed based on requirements for new cell wall material. This may indicate that a significant portion of the vesicles delivered to the apex does not succeed in contacting the plasma membrane for delivery of their contents. Therefore, we propose that more than one passage into the apex may be needed for many vesicles before they fuse to the plasma membrane and deliver their contents.

The dynamics of cellular organelles reveals important information about their functions and mutual interactions. The characterization and quantification of organelle dynamics requires microscopic observation of living cells. While tracking of larger organelles such as mitochondria, Golgi bodies, and vacuoles is relatively easy to achieve using optical microscopy, smaller organelles such as vesicles are below the resolution capacity of the optical microscope. This by no means renders their observation or tracking impossible. If the organelle in question is not packed densely, it can be tracked individually. However, problems arise if the population of identical organelles is densely packed, which is typically the case for secretory vesicles. In these situations, quantitative analyses of the speed and direction of vesicle movements are hampered by the fact that identification of a particular vesicle from one frame in a time-lapse series to the next is nearly impossible. Therefore, if densely packed, the small size of vesicles in combination with their significant movement rates necessitate both high-frequency imaging and advanced computational methods to study their dynamics.

Dense populations of vesicles moving rapidly are typical for plant cells with an active secretory mechanism and for rapidly growing cells. In plant cells, both cell growth and shape change require enlargement of the plasma membrane and expansion of the adjoining cell wall. To allow for an increase in cellular surface, additional material for both membrane and cell wall needs to be provided. These are delivered in the form of secretory vesicles. The contents packaged inside these vesicles consist of the precursor molecules that are added to the cell wall, whereas the membrane surrounding the vesicle is inserted into the expanding plasma membrane. To allow for the generation of shapes other than spheres, cellular expansion needs to be spatially controlled, thus requiring the localized addition of material at precisely controlled surface areas of the cell. Therefore, zones undergoing enhanced

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growth activity are also hot spots for vesicle trafficking. In no other plant cellular system is this process of precisely controlled vesicle delivery as impressively visible as in the rapidly growing pollen tube.

Pollen tubes are cellular protrusions formed by pollen grains upon contact with a receptive stigma. Their function is the delivery of the male gametes to the ovule located deep in the pistil of the receiving flower (Heslop-Harrison, 1987; Geitmann and Palanivelu, 2007; Wilsen and Hepler, 2007). Given that the principle of “first come, first served” rules, the growth process is extremely rapid and can achieve rates of up to 1 cm h⁻¹ (Booy et al., 1992). Contrary to diffuse growth, the growth process in pollen tubes is confined to the very apex of the cell. The metabolic activity of the pollen tube, therefore, is focused completely on the rapid delivery of cell wall precursor material and membrane toward this sole expanding region of the cell (Holdaway-Clarke and Hepler, 2003; Campanoni and Blatt, 2007; Chebli and Geitmann, 2007; Wilsen and Hepler, 2007). The intracellular transport processes must be highly controlled spatially and temporally to permit the coordinated delivery and release of precursor material. Given that dictyosomes are distributed over the entire cytoplasm and that the destination site for secretory vesicles is the hemisphere-shaped end of the cell, there must be an efficient long-distance transport system in place that coordinates the transfer from the site of synthesis to the site of release at the apex. Because of a quantitative difference between the amount of vesicle membrane around secretory vesicles delivering cell wall material and the amount of membrane required for the plasma membrane expansion (Picton and Steer, 1983), a portion of the membrane is recycled into the cytoplasm by endocytosis (Malhó et al., 2005). The vesicle population in growing pollen tubes, therefore, is composed of large numbers of both endocytotic and exocytotic vesicles, and it probably comprises more categories, as various types of molecules (polysaccharides, proteins) are exported and not all of the molecules transported in vesicles are destined for secretion.

In pollen tubes, organelle transport occurs along both microtubules and actin filaments (Cai et al., 1996, 2005; Moscatelli et al., 1997; Geitmann and Emons, 2000; Yokota and Shimmen, 2006; Romagnoli et al., 2007). However, while actin filaments are indispensable for cytoplasmic transport in these cells, microtubule inhibitors do not prevent either cytoplasmic streaming or pollen tube growth. The trajectories of larger organelles have been traced individually (Pierson et al., 1990; de Win et al., 1997, 1998, 1999) or in bulk (Lový-Wheeler et al., 2007) in living pollen tubes using different techniques. The movements of these organelles are related to the spatial configuration of the actin cytoskeleton that is arranged in longitudinal cables parallel to the long axis of the cell.

Due to the longitudinal arrangement of the actin cytoskeleton, the two principal directions of organelle movement are forward and rearward. Forward movement occurs mostly in the periphery, whereas rearward streaming is displayed along the central axis of the tube, creating a reverse fountain-like pattern (Iwanami, 1956). The turnaround point for larger organelles such as amyloplasts and vacuoles is at a significant distance before reaching the apex, while others such as mitochondria and endoplasmic reticulum reach the subapical region (Lový-Wheeler et al., 2007). Vesicles are presumed to follow the same tracks as the other organelles. However, contrary to the larger organelles, they enter the apical cytoplasm. They first reach the region defined by a conspicuous configuration of the actin cytoskeleton, the actin fringe. This structure consists of finer actin bundles located in the periphery of the cell and reaching partly into the hemisphere-shaped dome of the apex (Lový-Wheeler et al., 2005). Once they have reached this region, vesicles are presumed to flow into the apical cytoplasm of the cell. However, while transmission electron microscopic observations have clearly shown the accumulation of vesicles in the apical cytoplasm of pollen tubes, their small size and dense packing makes observation of their dynamics in the apex a challenge. With a typical diameter of 75 to 200 nm (Derksen et al., 1995), pollen tube vesicles are below the resolution capacity of the conventional optical microscope. Their bulk movements can be observed when using fluorescence label (Parton et al., 2001, 2003; Camacho and Malhó, 2003; Monteiro et al., 2005; Zonia and Munnik, 2008), but the quantification of their individual motion poses a problem. Nevertheless, attempts have been made to do so. Evanscent wave (or total internal reflection fluorescence) microscopy is able to overcome the resolution limit of the conventional optical microscope, but at present, available techniques limit the observation zone of the specimen to an optical slice with a thickness of only 300 nm adjacent to the plasma membrane, thus making the observation and quantification of global movement patterns in the entire apical region and especially at the extreme apex impossible. Total internal reflection fluorescence microscopy studies nevertheless provided information on the moving trajectories of individual vesicles close to the plasma membrane (Wang et al., 2006).

Here, we used two recent developments to circumvent the technical problems associated with the quantification of vesicle dynamics in dense populations and on distance scales larger than the nanometer range. To be able to monitor rapid movements, we used the Zeiss LSM 5 LIVE system, which permits extremely fast image acquisition compared with conventional confocal laser scanning microscopy. To quantify the movement of densely packed objects below the optical resolution limit, we applied spatiotemporal image correlation spectroscopy (STICS; Hebert et al., 2005). This allowed us to measure the average speed of vesicle movement in the different subcellular regions. We related this information to the cellular architecture in these regions and calculated the flow of vesicles in a three-dimensional space from transmission electron microscopy data. This approach has allowed us to make the first quantification of the vesicle traffic in growing pollen tubes.
RESULTS

Quantification of Vesicle Dynamics Using STICS

To reveal the pattern of vesicle streaming in growing pollen tubes, we added the lipophilic dye FM1-43 to growing pollen tubes and observed the cells with the Zeiss LSM 5 LIVE and Zeiss 510 Meta systems. The dye rapidly labels the plasma membrane and becomes internalized within a few minutes. The dye labels various types of intracellular membranes, and we cannot exclude that in the shank region organelles other than vesicles are marked. However, since we only apply the label for few minutes and observe the cells shortly after, the contribution of other organelles to overall movement patterns should be small. The intense staining of the cytoplasm in the apex of the tube is consistent with vesicles being one of the principal organelle populations being labeled (Fig. 1A). To further corroborate this, we applied simultaneous label with FM1-43 and MitoTracker. Mitochondria represent the population of larger organelles that reaches farthest into the tip region (Lový-Wheeler et al., 2007). Figure 1B clearly shows that there is no colocalization of FM1-43 and MitoTracker label in the apical and subapical regions, confirming that the intense FM1-43 staining in the apex corresponds to labeled vesicles. Remarkably, contrary to previously published images and our own images acquired with the 510 META system, the rapid scan rate in the LSM 5 LIVE system allowed us to show rapidly moving objects (likely vesicles and groups of vesicles) in the shank of the tube. The lower scan rates of the conventional 510 META mode and of other conventional setups used in the past probably prevent capturing these rapidly moving elements.

We used time-lapse imaging to monitor the vesicle movement at imaging rates of 10 or 18 frames s⁻¹ using the LSM 5 LIVE mode (Supplemental Movie S1). We then submitted the video material to STICS analysis. This method allows us to generate complete transport maps of vesicles within different regions of the cell even if the vesicle concentration was too high to perform single particle-tracking measurements. It is an extension of image correlation spectroscopy and image cross-correlation spectroscopy that relies on the complete analysis of both the temporal and spatial correlation lags for intensity fluctuations from a laser scanning microscopy image series (Hebert et al., 2005). This new approach allows the measurement of both diffusion coefficients and velocity vectors (magnitude and direction) for fluorescently labeled objects in living cells through monitoring of the time evolution of the full space-time correlation function.

Figure 1C shows a vector map overlying the micrograph of a corresponding pollen tube. The direction and size of the vectors clearly indicate that the fastest movements are at the periphery of the tube with the direction being forward and in the center of the tube where organelles return to flow rearward from the tip toward the shank of the tube. This streaming pattern corresponds to the general principle of the reverse fountain pattern previously described for pollen tubes of this species. While previous studies were based on the observation of larger organelles, our material proves that, not surprisingly, vesicles follow the same spatial pattern. However, vesicles actually reach the extreme apex, contrary to larger organelles such as amyloplasts, which are known to turn around at a distance of 10 to 20 μm from the apex. The fastest movement rates observed with STICS in the apical region were approximately 1.1 μm s⁻¹.

Vesicles Are Released into the Apical Cytoplasm in an Annulus-Shaped Region

The movement of secretory vesicles in pollen tubes has generally been presumed to be a forward movement leading to an accumulation of vesicles in the tip followed by contact with the apical membrane to liberate their contents. This concept implies that the densely accumulated vesicles forming the “inverted cone” in the pollen tube tip are “waiting for their turn” to undergo exocytosis, or, in other words, they should show an overall tendency for forward movement toward the apical plasma membrane. However, our STICS analysis revealed that forward movement occurs rather in the periphery, whereas in the center of the tube apex streaming is rearward. This is consistent with observations made by Parton et al. (2001). In the region corresponding to the inverted cone, our STICS analysis at a window size of 32 × 32 pixels and on time-lapse series of 100 frames did not reveal much movement (Fig. 1C). It is known that larger organelles such as amyloplasts occasionally enter the apical region, where they move more erratically and on shorter distance scales compared with their linear, actin-myosin-driven movements in the shank (de Win et al., 1998, 1999). Therefore, we suspected that putative short and erratic vesicle motions were not discernible when using the present STICS settings. This is due to the fact that the direction and magnitude of the flow must be sufficiently homogeneous within a region of analysis in order for the correlation functions of the STICS to have a well-defined Gaussian shape (Fig. 1D). In the case of short-range erratic movement within an analysis window, the peaks of the correlation functions become impossible to reliably locate and track through the image time series. To better characterize vesicle movement in the apex, we reduced the window size for STICS analysis to 16 × 16 pixels and the length of the time-lapse series to 50 frames. As a result, no single vector map reflects the overall motion patterns, but we were able to capture small waves of vesicle motion in individual maps. Figure 1, E to G, provides three
Figure 1. STICS and FRAP analyses of growing lily pollen tubes labeled with the lipophilic styryl dye FM1-43. A, Fluorescence micrograph showing a single optical section acquired in the LIVE mode of the Zeiss LSM 5. The strong label in the inverted cone at the apex of the tube indicates that vesicles are labeled intensely with the dye. The corresponding time-lapse series is provided as Supplemental Movie S1. Bar = 5 μm. B, Fluorescence micrograph superimposed on a corresponding DIC image of a pollen tube simultaneously labeled with FM1-43 (green) and MitoTracker (red). Mitochondria represent the population of larger organelles that reach farthest into the apical region of growing pollen tubes. The absence of colocalization confirms that in the apical region, the FM1-43 signal corresponds to labeled vesicles. Bar = 5 μm. C, STICS analysis of movements of FM1-43-labeled organelles in the cytoplasm of a lily pollen tube. The vector map overlying the micrograph reveals the direction and magnitude of vesicle movement.

Vesicle Flow in Growing Pollen Tubes
examples that illustrate such small waves of localized motion. Figure 1E reveals a strong component of vesicle displacement originating from a cortical region in the shoulder of the hemisphere-shaped apex in the direction of the center of the apex. Figure 1F shows a wave of rapid rearward movement in the central region of the inverted cone. Figure 1G shows an outward motion in the subapical region that might indicate a direct recirculation of vesicles coming from the tail of the inverted cone back into the forward stream (also shown in Supplemental Movie S2). Together, these observations confirm the general trend for rearward movement in the inverted cone.

When comparing the movement patterns with the configuration of the cytoskeleton in the pollen tube (Lovvy-Wheeler et al., 2005), it seems that transport into the apex occurs along actin filaments forming the subapical fringe from which they are released into the cytoplasm, filling the extreme tip of the apex. This region of the cytoplasm is known to be almost devoid of cytoskeletal elements, and vesicles might just follow a hydrodynamic flow once they are released from the cytoskeleton. This “release zone” forms an annulus located at the proximal end of the actin fringe. To provide further evidence for this release zone, we performed FRAP analysis on growing pollen tubes. After allowing pollen tubes to take up FM1-43, excess dye present in the medium was removed and the entire apex (both cytoplasm and plasma membrane) was photobleached along a length of 15 to 20 μm, followed by observation of the subsequent fluorescence recovery. The photobleached area had a size of approximately 200 to 300 μm², thus necessitating bleaching times between 30 and 90 s at highest laser intensity. No differences in growth speed and cytoplasmic streaming occurred after this treatment, as observed with bright-field optics. Several subsequent FRAP experiments could be performed on a single tube without affecting growth. During recovery, the overall apical membrane did not recover the characteristic signal of plasma membrane labeling in the presence of external FM1-43, indicating that recovery of label in the apical cytoplasm was exclusively due to vesicle movement within the cell and not to the incorporation of residual dye from the germination medium (compare Fig. 1, H and I). After bleaching, the first visible signal was recovered between 1 and 20 s after photobleaching, depending on FM1-43 incorporation into the cell, laser intensity during image acquisition, and photobleaching strength (two or three scans).

Recovery of fluorescence in the apical inverted cone occurred gradually in the entire cone, in most cases clearly progressing from the putative annular release zone in the direction of the center and then toward the tail end of the cone. Many pollen tubes exhibited a clear initial accumulation of label in the shoulder of the apex, corresponding to the putative release zone, before the label spread to the rest of the cone (Fig. 1H; Supplemental Movie S3).

Vesicle Dynamics in the Vicinity of the Plasma Membrane

Despite the initial uptake into the endocytic pathway, it has been suggested that styryl dyes eventually make their way into the exocytic pathway (Read and Hickey, 2001; Bolte et al., 2004). In pollen tubes, this notion was based on the observation that the dye intensely labels the apical inverted cone population of vesicles (Parton et al., 2001, 2003; Camacho and Malhó, 2003; Monteiro et al., 2005; Zonia and Munnik, 2008). However, no evidence is available at present to

Figure 1. (Continued.)

speed of particle motion in the pollen tube as analyzed over a 10-s period. The dominant motions occur forward at the periphery of the tube and rearward in the center of the tube. Numbers on color lookup tables are movement rates in μm s⁻¹. Bar = 10 μm. D, Example of the evolution of the correlation function corresponding to the vector marked with a magenta dot in the left panel. The five images at right correspond to the intensity of the two-dimensional correlation function calculated for the lags indicated (numbers in seconds). They reveal the shift in the position of the peak (dark red) as well as a broadening of its waist, resulting from a certain degree of random movement within the assessed region. Note that due to the mathematical calculations used in STICS, the vector indicates a direction opposite to the movement of the peak. Window size, 32 × 32 pixels. Bars = 1 μm. E to G, STICS analysis of vesicle movements in the cytoplasm of a pollen tube apex. Vector maps were obtained using 16 × 16 pixel windows and sequences of 50 (C) or 100 (E and G) frames. Each image shows individual small waves of vesicle motion. E, Strong motion from the shoulder of the apex into its center (arrow). F, Prominent motion within the inverted cone in the direction of its tail (arrow). G, Outward motion from the cone to the periphery of the tube in the subapical region (arrows). Numbers on color lookup tables are movement rates in μm s⁻¹. Bars = 5 μm. H, FRAP analysis of vesicle dynamics in the apex of a growing pollen tube. The time-lapse series shows a pollen tube labeled with FM1-43 after removal of the dye in the medium (first frame) and at various times during recovery after photobleaching. Numbers indicate time in seconds after photobleaching. During recovery from photobleaching, there is an initial accumulation of label in the shoulders of the apex. The complete time-lapse series is available as Supplemental Movie S3. Bar = 5 μm. I, Fluorescence micrograph showing a single optical section acquired in the 510 Meta mode. FM1-43 was not removed prior to imaging, revealing intense label of the plasma membrane. Bar = 5 μm. J, Fluorescence micrographs and corresponding DIC images of a pollen tube changing growth direction during FRAP analysis. The images correspond to the graph shown in Figure 2D. Numbers correspond to time in seconds after photobleaching. Images were acquired with the Zeiss 510 Meta system. Arrows in the DIC images indicate the growth direction. Arrows in the fluorescence images indicate the region of low fluorescence recovery at the extreme apex changing its position relative to the original growth direction. Bar = 10 μm.
actually prove that the label corresponds to that of vesicles destined for exocytosis. Given that the plasma membrane does not recover significant label intensity after FRAP (compare Fig. 1, H and I), the cone-shaped pool could contain a significant number of vesicles that are just circulating and not necessarily destined to liberate their contents. To show that the labeled vesicles reach the vicinity of the apical membrane, we quantified FRAP in the cortical region representing the membrane and the underlying 1 μm of cytoplasm. To study the spatiotemporal dynamics in this cortical region, it was divided into 10 connecting zones (Fig. 2A). Analysis of the mean pixel intensity in each of the cortical zones showed that fluorescence recovery in zones closer to the extreme tip (zones 1/−1) was time delayed compared with that in zones farther away (Fig. 2, B and C). While zones 5/−5 generally remained low in fluorescence intensity, the first increase during recovery was observed in zones 4/−4 or 3/−3 and subsequently progressed toward the extreme tip. Fluorescence intensity in the cortical zones reached a plateau between 30 and 120 s after bleaching. Remarkably, zones 1/−1 generally did not recover the same level of fluorescence intensity as the adjacent zones (Fig. 2C). In tubes that changed growth direction during the recovery period, this central zone, characterized by lower label intensity, shifted to the side of the new growth direction (Figs. 1J and 2D).

Turnover Dynamics at the Apex Determined by FRAP Analyses

Vesicles labeled with FM1-43 are clearly delivered to the tip in the periphery of the cell, and most of them seem to flow back in the center. It is impossible in this experimental setup to determine whether they flow back with or without having contacted the apical membrane and liberated their contents or whether they actually are secretory vesicles. However, the vesicles move in a rather precisely determined three-dimensional space that has an entry region in the periphery and an exit region in the center. Therefore, we were interested in determining the turnover rate of this apical vesicle pool in order to compare it with the required number of secretory vesicles that is needed to sustain apical growth in the pollen tube.

To characterize the turnover rate within the apex, we performed FRAP analysis as described above and

Figure 2. FRAP analysis of the cortical region of a growing pollen tube. A, Subdivision of the cortical and central regions of the pollen tube apex into zones. In this fluorescence micrograph, all pixels that were not completely black were given a value of 255 (red) to illustrate that no background noise was observed outside of the tube after FM1-43 labeling and subsequent removal of the dye from the medium. Inset, Corresponding DIC image. Bars = 10 μm. B to D, Three examples of recovery of fluorescence intensity in the cortical zones identified in A. B, The recovery of fluorescence is time delayed between the different sections, with the central sections being last to initiate recovery. C, Fluorescence intensity in the extreme apex (zones 1/−1) does not recover the same fluorescence intensity as the adjacent zones. D, Example of a pollen tube undergoing a change of growth direction during recovery. The central region of slow and incomplete fluorescence recovery shifts from zones 1/−1 to zones 1/2.
quantified the time required for the fluorescent label in the inverted apical cone to reach a plateau. To do so, fluorescence intensity in three circular zones at fixed positions on the median axis of the pollen tube was quantified (Figs. 2A and 3). Recovery occurred progressively from zone C1 (closest to the extreme tip) to zone C3 (in the tail of the inverted cone), consistent with a general flow in a rearward direction in this central region. Analysis of the correlation between the dynamics of the fluorescence intensity in these three zones revealed that clusters of vesicles seemed to move through them with a delay of 2 to 4 s, corresponding to a speed of between 1 and 2 \( \mu \text{m s}^{-1} \) given the distance of 4 \( \mu \text{m} \) between the centers of the circular zones. We then plotted the time necessary for fluorescence intensity in region C2 to reach a plateau versus the growth rate of the individual tubes. Despite considerable variation, tubes with higher growth rates seemed to have shorter turnover rates, consistent with the need for elevated amounts of secreted cell wall material (Fig. 3B).

**Theoretical Vesicle Turnover Rates**

Next, we wanted to compare the observed FRAP recovery rates with the vesicle turnover rates that can be calculated based on pollen tube geometry, vesicle dimensions, and the resulting need for cell wall material. Previous attempts to calculate the vesicle turnover rate in the apical region of growing pollen tubes have provided important information on the speed of the transport processes involved. However, the available data cannot be used for this study. Picton and Steer (1983) used *Tradescantia virginiana* and Derksen et al. (1995) used tobacco (*Nicotiana tabacum*), both of which produce pollen tubes with a diameter corresponding to one-third or less of the diameter of pollen tubes from *Lilium*. van der Woude and Morré (1968) used lily (*Lilium longiflorum*) for their calculations, but the pollen tubes were fixed chemically and the resulting electron micrographs do not provide accurate information on vesicle size and distribution, as is evident upon comparison with more recent images obtained with rapid freeze fixation (Lancelle and Hepler, 1992).

Here, we used electron micrographs of median longitudinal sections of pollen tubes that were rapid freeze fixed and freeze substituted. To quantify the spatial and temporal motion patterns of the secretory vesicles, we calculated the following parameters.

**Vesicle Requirement to Sustain Growth**

The continuously elongating pollen tube requires the deposition of cell wall material. The volume of newly formed cell wall can be calculated as

\[
\Delta V_w = \Delta L \times \pi (R_o^2 - R_i^2)
\]

with \( \Delta L \) being the increase in pollen tube length, \( R_o \) the outer radius of the cylindrical pollen tube shank, typically 8.3 \( \mu \text{m} \), and \( R_i \) the inner radius of the pollen tube. \( R_i \) can be calculated from

\[
R_i = R_o - T
\]

with \( T \) being the thickness of the pectinaceous layer of the cell wall, typically 170 nm in the subapical region of the cell.
\(\Delta L\) can be calculated from
\[
\Delta L = G \times t
\]
with \(G\) being the growth rate, typically 7 \(\mu\text{m} \text{ min}^{-1}\), and \(t\) being time.

We thus obtain a typical value of \(\Delta V_w = 61.42 \mu\text{m}^3 \text{ min}^{-1}\).

The elongating tube also requires the exocytotic insertion of new membrane material, the surface of which can be calculated with
\[
\Delta S_M = \Delta L \times 2\pi \times R_i
\]
yielding a value of 357.58 \(\mu\text{m}^2 \text{ min}^{-1}\).

The volume of the cell wall material delivered in an individual secretory vesicle can be calculated by
\[
V_V = \frac{4}{3} \pi R_v^3
\]
with \(R_v = 85\) nm being a typical radius of a vesicle, thus yielding \(V_V = 0.00257 \mu\text{m}^3\).

The membrane surface delivered by an exocytotic vesicle is calculated to be 0.09079 \(\mu\text{m}^2\) with
\[
S_V = 4\pi R_v^2
\]

The number of vesicles required to allow for the necessary increase in plasma membrane surface at the growing tip can thus be calculated to be 3,939 \(\text{min}^{-1}\) using
\[
\Delta N_M = \frac{\Delta S_M}{S_V}
\]

It is not known whether the cell wall precursor material delivered in exocytotic vesicles changes its volume upon insertion into the existing cell wall. Both an increase (through water uptake) and a decrease (through optimal alignment and stretching of polymers) could be conceived. Therefore, for the purpose of this model, we assume that no change in volume takes place. This assumption is supported by the observation that the electron density of secretory vesicles and the cell wall is very similar in cryofixed pollen tubes from lily and other species (Lancelle and Hepler, 1992; Derksen et al., 1995). Then, the number of vesicles required to provide the cell wall material necessary for elongation can be calculated to be 23,899 \(\text{min}^{-1}\) with
\[
\Delta N_W = \frac{\Delta V_w}{V_V}
\]

This leaves a net surplus of \(\Delta N_C = \Delta N_W - \Delta N_M = 19,960\) vesicles \(\text{min}^{-1}\), the cell wall precursor contents of which need to be delivered to the apex but whose membrane is not required for pollen tube elongation.

**Vesicle Dynamics in the Apical Region**

The apex of a growing lily pollen tube can be approximated as having the shape of a half sphere capping a cylinder. The volume of the bleached region, therefore, can be calculated as
\[
V_A = \frac{2}{3} \pi R_i^3 + \pi R_i^2 L_B
\]
with \(L_B\) being the length of the cylindrical part of the tube that gets bleached during FRAP analysis (Fig. 4).

To determine the number of vesicles in the bleached apex, we counted the number of vesicles visible in a median section of the apical 20 \(\mu\text{m}\) of a pollen tube \((N_{MS})\) and calculated the approximate volume of a median section assuming a thickness of 90 nm (the typical thickness of an ultrathin section used for transmission electron microscopy)
\[
V_{MS} = 90\text{nm} \times \frac{1}{2} \pi R_i^2 + 2R_i \times L_B
\]

The number of vesicles in the volume of the bleached apex can then be calculated as
\[
N_A = \frac{N_{MS} \times 90\text{nm}}{V_{MS}} \times V_A
\]

The calculation of \(N_A\) resulted in an average of 81,247 \(\pm 11,490\) (\(n = 5\)).

The turnover time for the vesicle population in the apical inverted cone can then be calculated as
\[
t = \frac{N_A}{\Delta N_C} = 244\text{ s}
\]
or approximately 4 min.

The curve for the theoretical turnover times versus the growth rate was then added to the graph in Figure 3B. Comparison with the measured values showed that while a similarly shaped curve can be fitted through the experimental data, despite considerable

![Figure 4. Transmission electron micrograph of a median section of a freeze-fixed pollen tube illustrating the cylinder/hemisphere-shaped geometry of the tube and the distribution of vesicles in the shape of an inverted cone. Ri, Radius of the hemisphere-shaped apex and of the cylindrical shank; Lb, length of the cylindrical shank bleached during FRAP analysis. Bar = 3 \(\mu\text{m}\).](image-url)
scatter, the experimental values for turnover time are significantly lower than those calculated based on the requirement for cell wall material.

DISCUSSION

Vesicle Trafficking and Material Requirements for Growth

Intracellular transport processes are complex and occur between a variety of organelles and cellular compartments. A very important step in the intracellular membrane trafficking is the transport involved in the production of extracellular substances, such as export proteins and cell wall polysaccharides. In few plant cells is this trafficking process as vigorous as in the rapidly growing pollen tube, a cellular protrusion that requires the continuous secretion of cell wall material to provide the elongating apex with cell wall polysaccharides, proteins, and plasma membrane. Our calculations show that in lily pollen tubes, each minute tens of thousands of vesicles must deliver their contents to the apex to provide the material necessary to form the elongating wall. However, only a small fraction of these vesicles is necessary to produce the additional plasma membrane surface required to sustain growth. According to our calculations, only the membrane of 16.5% of the secretory vesicles reaching the apex will thus be incorporated permanently into the plasma membrane of growing lily pollen tubes. Despite a significant difference in total vesicle numbers between species (due to slower growth rates, different vesicle diameters, wall thickness, and smaller tube diameters), this percentage value is actually very close to that of 13.1% determined for tobacco (Derksen et al., 1995). For the latter species, it has been proposed that the excess membrane is reincorporated by clathrin-mediated endocytosis. Evidence for endocytosis in the apical region of the pollen tube is based on the uptake of cell-impermeant fluorescent markers (O’Driscoll et al., 1993), the presence of clathrin in this region of the cell (Derksten et al., 1995; Blackbourn and Jackson, 1996), and the internalization of fluorescent phospholipids (Lisboa et al., 2008), steryl dyes (Moscataelli et al., 2007; Zonia and Munnik, 2008), and nanogold particles (Moscataelli et al., 2007). However, the observed number of coated pits in the pollen tube apex is not sufficient to account for the calculated number of endocytosis events (Derksen et al., 1995). Therefore, alternative mechanisms, such as smooth vesicle or kiss-and-run endocytosis, are likely to take place as well. During kiss-and-run exocytosis, the vesicle membrane does not actually collapse into the plasma membrane but simply releases its contents through a short-lived fusion pore before detaching and reentering cytoplasmic circulation (Stevens and Williams, 2000). Recently, Balaji and Ryan (2007) have shown that exocytosis of synaptic vesicles is coupled to endocytosis by a stochastic process of approximately 14 s. Given the huge throughput of vesicles at the pollen tube tip, these mechanisms seem to be worth investigating. A kiss-and-run mechanism could also explain the phenomenon that the plasma membrane does not regain significant fluorescence after photobleaching, since the fluorescently labeled vesicle membrane would not be inserted into the plasma membrane. Another phenomenon that could be explained by the extremely rapid kiss-and-run mechanism is the infrequency of exocytosis events captured in transmission electron sections of the apex, despite fixation by rapid freeze methods (Lancellle and Hepler, 1992).

Spatial Organization of Vesicle Dynamics in the Apex

Organelle and vesicle movements in pollen tubes are largely based on an actin-myosin system (Geitmann and Emons, 2000; Vidali and Hepler, 2001). In lily pollen tubes, actin filaments are organized in long arrays oriented parallel to the longitudinal axis of the cell. In the apical region, fine actin filaments form a cortical fringe (Lovly-Wheeler et al., 2005; Cárdenas et al., 2008). Our STICS analyses show that vesicles are delivered into the apical pool at a position that corresponds exactly to the proximal end of the actin fringe. It is unknown how vesicles move on from there and how they are sorted. How do vesicles that have yet to contact the plasma membrane find their way and avoid being swept away by the wave of vesicles returning from the membrane and reversing their flow direction to stream back toward the center of the tube? We hypothesize that precisely targeted, actin-guided delivery in very close vicinity to the membrane of the shoulder of the apex increases the chance of secretory vesicles releasing their contents right there (Fig. 5). Other vesicles that do not undergo exocytosis and those that are generated by endocytosis, probably in the extreme apex, then flow into the cone-shaped pool of vesicles by mass flow, from where they are ushered back in the shank region of the tube.

An essential consequence of this model is the fact that vesicles delivered to the apex seem to hover in an annulus-shaped region at the shoulder of the apex and not in the extreme apex. While our images did not allow the observation of actual exocytosis events, the initial accumulation of vesicles in a ring-shaped zone after FRAP may suggest that the main exocytosis activity takes place there as opposed to the extreme apex. This concept is further supported by the finding that fluorescent label in the extreme apex (zones 1/ −1) does not recover as much fluorescence intensity as label in the annulus. The fact that this central zone of lower fluorescence is maintained over longer times is indicative of endocytosis taking place there (i.e. uptake of unlabeled plasma membrane). This concept is consistent with the observation that negatively charged nanogold particles are internalized in the apical region of growing tobacco pollen tubes (Moscataelli et al., 2007).
The existence of spatially defined regions of exocytosis and endocytosis activity is further corroborated by the observation that the fluorescence profile of the apical cortex changes orientation when the pollen tube changes its growth direction. Similar changes in orientation of the cone-shaped vesicle pool in its entirety were observed in pollen tubes of *Agapanthus* (Camacho and Malhó, 2003) and tobacco (Moscatelli et al., 2007). Remarkably, an annulus-shaped secretion region would fit theoretical models for tip growth that propose that surface expansion is faster in an annulus-shaped region around the extreme apex of growing root hairs (Shaw et al., 2000). The spatial colocalization of the vesicle accumulation region and theoretically computed surface expansion with the proximal end of the actin fringe suggest that the latter controls the former (Cárdenas et al., 2008).

It is interesting that the concept of an annulus-shaped secretion zone is unlikely to be a general mechanism in all tip-growing cells. Fungal hyphae have an outer geometry that is very similar to that of pollen tubes and root hairs, but they are known to possess a Spitzenkörper in their apical cytoplasm, a structure consisting of vesicles and cytoskeletal elements from which secretory vesicles are presumed to be sent to the entire surface of the apical membrane (Bartnicki-Garcia et al., 1989; Bartnicki-García, 1990, 2002; Tindemans et al., 2006). Detailed investigations of the directionality of vesicle movements in these cells are not available, however, and might make for interesting comparison.

**Identity of Vesicles in the Apical Pool**

According to the flow patterns observed with STICS and FRAP analyses, the vesicles in the cone-shaped apical pool should not be destined for fusion with the apical plasma membrane but are already coming from there. Therefore, it is puzzling that most of the vesicles forming the inverted cone seem to contain cell wall precursor material, given their fibrillar appearance—or so they had been interpreted (van der Woude et al., 1971; Picton and Steer, 1983; Heslop-Harrison, 1987; Derksen et al., 1995). Immunogold labeling has shown that secretory vesicles in pollen tubes contain pectins (Geitmann et al., 1995; Li et al., 1995), but none of the available micrographs with immunolabel shows a median section of the pollen tube apex that would have allowed us to verify the contents of the vesicles in the inverted apical cone. However, the significantly higher turnover rate measured after FRAP compared with that calculated from pollen tube geometry might provide an explanation. It suggests that many vesicles, despite containing cell wall precursors, simply fail to fuse with the apical membrane and thus are circled rearward into the shank before potentially returning into the forward stream to obtain a second chance to liberate their contents. The unsuccessful passage of the

![Figure 5. Schematic drawing illustrating the principal directions of vesicle flow in the apical region of a pollen tube. Following delivery into the apical region on the actin filaments forming the cortical fringe, vesicles are released into the apical cytoplasm in an annulus-shaped zone. Some of the vesicles that succeed in contacting the plasma membrane fuse with it and undergo exocytosis, whereas a significant portion might deliver their contents by a kiss-and-run mechanism. Vesicles that do not succeed in contacting the plasma membrane stream rearward within the cone-shaped vesicle pool. Many of these vesicles are recirculated back into the forward stream immediately in the subapical region. At the extreme apex, the principal activity is endocytosis, either by a clathrin-mediated or a clathrin-independent (smooth) mechanism. Clathrin-mediated endocytosis also takes place in a more distal region, based on observations by Derksen et al. (1995), Moscatelli et al. (2007), and Zonia and Munnik (2008). The position of the actin fringe is based on Lovy-Wheeler et al. (2005). Objects are not drawn to scale. For clarity, except for vesicles, no other organelle or the cell wall is drawn. The sketch of the tube at the bottom shows the position of the annulus-shaped release zone.](https://example.com/figure5.png)
apex followed by the recirculation via the cone and the subapex might explain that vesicles in the inverted cone contain cell wall material. However, a portion of the cone-shaped vesicle pool should nevertheless be of endocytotic nature. It is interesting in this context that vesicles containing fibrillar material were observed to be involved in nanogold internalization in tobacco pollen tubes, suggesting that endocytotic vesicles might contain recycled cell wall material (Moscatelli et al., 2007). Internalization of pectins has also been observed in somatic plant cells (Baluška et al., 2002, 2005) and has been cited in a theoretical model attempting to explain oscillatory pollen tube growth (Kroeger et al., 2008).

On the other hand, the hypothesis that most of the vesicles in the apical inverted cone are not destined for fusion with the apical plasma membrane is consistent with the finding that in tobacco, expression of GFP coupled to pectin methyl esterase, a protein secreted by the pollen tube, does not label the region of the inverted cone despite being successfully secreted to the apical cell wall (Bosch et al., 2005). Vesicles containing this protein might be more successful in delivering their loads upon first arrival in the apex, thus not requiring repeated passages through the cone. To elucidate the identity of vesicles in the inverted cone further, detailed analyses such as those done using electron tomography on vesicles in Chama rhizoids (Limbach et al., 2008) are warranted to determine the different types of vesicles in the apical region of pollen tubes.

Vesicle Movement and Turnover Rates

Speeds of individual organelles in pollen tubes of various species have been quantified to achieve up to several micrometers per second in the cylindrical shank of the tube. Maximum individual organelle velocity observed in pollen tube of lily was 0.6 μm s⁻¹ (Iwanami, 1956). Wang et al. (2006) observed two kinds of vesicle movements in the pollen tube of Picea meyeri: long-distance and short-distance motions, with average velocities of 1.93 and 1.09 μm s⁻¹, respectively. In vitro, the average speed of Golgi vesicles was measured to be 1.78 μm s⁻¹ on actin filaments, 0.22 μm s⁻¹ on microtubules, and 0.75 μm s⁻¹ in the presence of both microtubules and actin filaments (Romagnoli et al., 2007). These values are comparable to those we measured on lily pollen tubes using STICS, which were typically between 0.2 and 0.5 μm s⁻¹ but could reach up to 1.1 μm s⁻¹. In the apical region, no consistent streams of rapid vesicle movement were observed; rather, they were localized, short-lived waves. Therefore, the average speed of vesicle movement in the apex was rather low compared with organelle speed in the shank of the tube.

Despite the relatively slow movement of vesicles in the apex as measured with STICS, the time required for turnover of the apical vesicle population is astonishingly fast, significantly faster than the theoretical calculations. The most likely parameter that might explain the significant difference between theory and experiment is the assumption used in the theoretical calculations that all vesicles transported to the apex actually fuse with the apical membrane and deliver cell wall material to the growing tip. The observation that real-time measurements of vesicle turnover in the apex revealed higher rates suggests that a significant portion of the vesicles reaching the apical cytoplasm might not actually fuse with the plasma membrane but might stream back into the shank of the tube. Only a fraction of the vesicles arriving in the apical region releases cell wall material. One reason for this might be that a portion of the vesicles streaming into the tip is not of the secretory type and that their function might be other than the delivery of cell wall material. Alternatively, many vesicles that are destined for secretion might not come into contact with the apical membrane the first time around before being swept away by the rearward current. The observation of an outward movement in the subapical region is consistent with this hypothesis and suggests that these vesicles reenter the forward circulation immediately after leaving the inverted cone at its tail. The consequence is that the global vesicle turnover of the apical vesicle population is significantly higher than the theoretically established one, in order to ensure that sufficient numbers of vesicles are delivered that successfully release their contents.

MATERIALS AND METHODS

Plant Material

Pollen grains of lily (Lilium longiflorum) were harvested from plants grown in the greenhouses of the Montreal Botanical Garden. Pollen was packed in gelatin capsules, dried overnight on silica gel, and stored at −80°C. Pollen was rehydrated in a humid chamber for 30 min and transferred to germination medium containing 1 mM KNO₃, 130 nM Ca(NO₃)₂, 160 nM H₃BO₃, 10% Suc (w/v), and 5 mM MES buffer adjusted to pH 5.5.

Membrane Labeling

Membranes were fluorescently labeled by addition to the germination medium of FM-43 (Molecular Probes, Invitrogen) to a final concentration of 160 nM. After 5 min of labeling, pollen tubes were washed four times and re suspended in germination medium after the FM-43 labeling and washing steps.

Mitochondria Labeling

Mitochondria were fluorescently labeled by the addition of MitoTracker Red CMXRos (Molecular Probes, Invitrogen) at a final concentration of 50 nM to the germination medium after the FM-43 labeling and washing steps.

Confocal Laser Scanning Microscopy

Confocal laser scanning imaging was performed with a Zeiss LSM 510 META/LSM 5 LIVE/Axiovert 200 m system. The microscope was fitted with a Plan Apochromat 100×/1.4 oil differential interference contrast (DIC) objective. For FRAP experiments, we used the 488-nm line of the argon laser (maximal power, 30 mW; output set to 40%; transmission, 15%–30% for image
acquisition and 100% for photobleaching, and emission filter LP 575. For LIVE imaging, we used the 488-nm diode laser (maximal power, 100 mW) and emission filter LP 550. Image resolution was 512 × 512 pixels with 1 μm = 11.4 pixels in the META mode and 1 μm = 7.1 pixels in the LIVE mode.

Quantifications of FRAP were performed using Image J (http://rsb.info.nih.gov/ij/) and the Radial Profile Extended plugin (http://rsb.info.nih.gov/ij/plugins/radial-profile-ext.html) that integrates signal intensity in an area defined by a starting angle ± an integration angle. The tip was radially divided in 10 adjacent radial zones of 18° vertex angles originating from the center of a circle fitting the shape of the pollen tube tip periphery. The sections covered a region starting slightly outside of the cell reaching 1 μm into the cytoplasm (Fig. 2A). The section profile was positioned manually in each analyzed image. The fluorescent signal in each zone was integrated with the plugin function “Plot and Calculate ROI Radius” and divided by the number of pixels to obtain the mean pixel intensity. The signal was also quantified in three circular zones, C1, C2, and C3, positioned on the median axis of the pollen tube tip: C2 is localized at the intersection of the radii described previously; C1 is localized at half the distance between C2 and the tip, on the median axis of the tube; and C3 is symmetrically positioned on the median axis on the shank side of C2 (Fig. 2A).

Transmission Electron Microscopy

Pollen tubes grown for 2 h were collected on formvar-coated wire loops and rapidly frozen as described previously (Lancelle and Hepler, 1992). The samples were allowed to freeze substitute at ~80°C in 2% OsO₄ in acetone for 36 h, warmed to room temperature over 5 to 6 h, and then transferred to methanol. They were stained en bloc for 2 h at room temperature in 5% uranyl acetate in methanol, rinsed in methanol, and then transferred back to acetone before infiltration in Epon-Araldit resin and embedding between release-coated glass slides. Cells for sectioning were selected with the light microscope. These sections were stained for 3 min in Reynolds’ lead citrate before examination on a JEOL 100CX electron microscope operating at 80 kV.

STICS

STICS is an extension of other image correlation techniques that were developed to measure the directed transport or flow of proteins inside living cells (Hebert et al., 2005; Brown et al., 2006). The analysis is applied directly to an image time series recorded using a fluorescence microscope, such as the confocal laser scanning microscopy image series reported in this work. STICS relies on calculating the complete space-time correlation function of the intensity fluctuations between images in the time series. The generalized space-time correlation function can be approximated by calculating the spatial correlation between pairs of images in the time series as a function of time separation between image pairs:

\[
r'(\xi, \Delta t) = r'(\Delta x, \Delta y, \Delta t) = \frac{1}{N-\varepsilon} \sum_{i=1}^{N-\varepsilon} b(i)(i(x, y, t)) \times H_1(f)\left(\frac{r(i(x, y, t)) - H_1(f)}{t}\right)
\]

where \(N\) is the total number of frames in the image series, \(s\) is the image lag or shift variable and is simply the number of frames between pairs of images that are being correlated, \(\langle i \rangle_0\) and \(\langle i \rangle_n\) are the average pixel intensities for the \(k\) and \(k + s\) image frames, respectively, \(b(i(x, y)) = \langle i(x, y)\rangle - \langle i \rangle_0\) is the intensity fluctuations at pixel position \((x,y)\) in frame \(k\), and similarly for the space-time-shifted pixel fluctuation \(\delta i(x + \Delta x, y + \Delta y, k + s)\), where \(\Delta x\) and \(\Delta y\) are discrete pixel shift variables for the spatial correlation. The angular brackets in the numerator represent an ensemble average over all pixel fluctuations for pairs of images separated by \(s\) frames. Note that the discrete time shift, \(\Delta t\), is simply the product of the image lag variable and the image frame acquisition time: \(\Delta t = s \times \Delta t_{frame}\). Similarly, the spatial lag variables are the product of the discrete pixel shifts with the pixel diameter \(\delta p\): \(\xi = \Delta x \delta p\) and \(\eta = \Delta y \delta p\).

In the STICS calculation, the correlation function is first calculated for every frame in the series, with \(\Delta t = 0\) (i.e. \(s = 0\)). For every image frame, this correlation function will appear as two-dimensional Gaussian function with its peak centered at the zero spatial lags position \((\xi = 0, \eta = 0, s = 0)\) because nothing has moved in zero time. Similarly, the correlation functions for all pairs of images separated by lag time \(\Delta t\) are calculated for \(s\) ranging from 1 to \(N - 1\). If the spatial distribution of labeled particles has changed between frames, due to the movement of particles, the Gaussian peak of the correlation function will change in location and/or width. For a flowing population of particles, the spatial correlation Gaussian peak will stay constant in width, but its peak position will shift over time to a new position \((\xi = -v_x \Delta t, \eta = -v_y \Delta t)\), where \(v_x\) and \(v_y\) are the \(x\) and \(y\) velocities of the flowing particles; whereas for diffusion, the Gaussian correlation peak will remain centered (i.e. it will not translate in space) but it will broaden and decrease in amplitude over time. In the case of both diffusion and flow, the position of the peak shifts while its waist broadens. A series of filters is used when analyzing the shape of the correlation functions for every lag time, such that tracking is terminated if the peak of the Gaussian function cannot be reliably located or if the width of the peak reaches a certain threshold. It is the time evolution of this Gaussian correlation function of intensity fluctuations that we track to obtain the velocity vectors of the flowing vesicles inside the pollen tubes.

Immobile Population Removal

The presence of an immobile population of particles in the region of analysis can introduce an unwanted static contribution to the correlation functions that can mask the dynamics of transport we wish to measure. To overcome this, we filter the image time series prior to calculating the correlation functions, such that the immobile components are removed from the raw images (Brown et al., 2006). This is done in frequency space, where the low-frequency components of each pixel’s intensity profile are removed before reverting back to the time domain. The corrected intensities are then given by

\[
\tilde{r}(x, y, t) = F_r^{-1}\left\{F_r\left(\sum_{i=1}^{N-\varepsilon} b(i)(i(x, y, t)) \times H_1(f)\right)\right\}
\]

where \(F_r^{-1}\) is the (inverse) Fourier transform with respect to variable \(s\), \(H_1(f)\) is the Heaviside function, which is 0 for \(f < 1/T\) and 1 for \(f > 1/T\), \(f\) is the pixel frequency in time, and \(T\) is the total acquisition time of the image time series.

The software used for STICS analysis is coded in Matlab and called STICSGui v0.21 and was developed by the Wiseman Research Group at McGill University. It is available upon request as a stand-alone executable.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Movie S1. Vesicle dynamics in a growing pollen tube labeled with FM1-43 observed with the Zeiss LSM 5 LIVE setup.

Supplemental Movie S2. Vesicle dynamics in a growing pollen tube labeled with FM1-43 showing vesicle motion from the tail of the inverted cone toward the periphery of the tube back into the cortical forward stream.

Supplemental Movie S3. FRAP analysis of vesicle dynamics in the apex of a growing pollen tube showing an initial accumulation of label in the shoulder of the apex.

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