Pulsatile influxes of H+, K+ and Ca2+ lag growth pulses of *Lilium longiflorum* pollen tubes

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Fluxes of H+, K+ and Ca2+ were measured with self-referencing ion-selective probes, near the plasma membrane of growing *Lilium longiflorum* pollen tubes. Measurements from three regions around short, steady-growing tubes showed small, steady influx of H+ over the distal 40 μm and a region of the tube within 50-100 μm of the grain with larger magnitude efflux from the grain. K+ fluxes were immeasurable in short tubes. Measurements of longer tubes that were growing in a pulsatile manner revealed a pulsatile influx of both H+ and K+ at the growing tip. The average fluxes at the cell surface during the peaks of the H+ and K+ pulses were 489±81 and 688±144 pmol cm⁻² second⁻¹, respectively. Growth was measured by tracking the pollen tips with a computer vision system that achieved a spatial resolution of approximately 1/10 pixel. The high spatial resolution enabled the detection of growth, and thus the changes in growth rates, with a temporal sampling rate of 1 frame/second. These data show that the H+ and K+ pulses have a phase lag of 103±9 and 100±11 degrees, respectively, with respect to the growth pulses.

Calcium fluxes were also measured in growing tubes. During steady growth, the calcium influx was relatively steady. When pulsatile growth began, the basal Ca2+ influx decreased and a pulsatile component appeared, superimposed on the reduced basal Ca2+ flux. The peaks of the Ca2+ pulses at the cell surface averaged 38.4±2.5 pmol cm⁻² second⁻¹. Longer tubes had large pulsatile Ca2+ fluxes with smaller baseline fluxes. The Ca2+ influx pulses had a phase lag of 123±9 degrees with respect to the growth pulses.

Key words: Pulsatile growth, H+ influx, K+ influx, Ca2+ influx, Computer Vision, Pollen tube, *Lilium longiflorum*

INTRODUCTION

The rapid, directed growth of the pollen tube is a central event in the sexual reproduction of higher plants. The addition of cell wall and cell membrane components to one elongating end (Lancelle and Hepler, 1992; Larson, 1965; Rosen et al., 1964) produces a tube that penetrates the stigma and grows through the style in order to transport the sperm cells to the embryo sac at the base of the style. In vitro growth of lily pollen tubes occurs at a steady rate averaging about 0.2 mm/second, until tubes reach about 1 mm in length. At this time growth becomes pulsatile, with growth rates oscillating between 0.1 and 0.4 mm/second (Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997; Pierson et al., 1996) and an average period between 23 (Holdaway-Clarke et al., 1997; Pierson et al., 1996) and 42 seconds (Messerli and Robinson, 1997). Pulsatile growth appears to be the dominant mode of extension of rapidly extending tip-growing organisms. López-Franco et al. (1994) have shown that fungal hyphae from seven genera all grow in a pulsatile manner. Likewise, pollen tubes from a number of plants that have been studied with adequate spatial and temporal resolution have been found to grow in an oscillatory manner; these include, in addition to lily, pollen tubes from *Nicotiana tabacum, Petunia hybrida, Gasteria verrucosa* (Pierson et al., 1995) and *Zea mays* (M. A. Messerli and K. R. Robinson, unpublished observation). It appears that there are constraints on growth that require it to be pulsatile; presumably, these constraints arise from the need to maximize growth rates. We are interested in understanding the physiological mechanisms that are involved in pulsatile growth and the feedback system that must be present to initiate and control the oscillatory behavior.

Because of its central role in secretion in general and in pollen tube growth in particular, considerable attention has been focused on cytosolic Ca2+. External Ca2+ is necessary for pollen tube growth (Brewbaker and Kwack, 1963; Picton and Steer, 1983). Advances in optical methods for imaging Ca2+ have allowed the cytoplasmic spatial and temporal distribution of Ca2+ in growing pollen tubes to be known in considerable detail. Pollen tubes have a tip-high gradient of Ca2+ that is necessary for growth; if that gradient is disrupted by any means, growth ceases (Miller et al., 1992; Rathore et al., 1991). More recently, it has been shown that oscillations of Ca2+ at the tip are superimposed on the steady gradient (Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). The Ca2+ oscillations have the same frequency as the growth oscillations and are nearly in
phase with growth; that is, the peaks of tip-localized Ca$^{2+}$ nearly coincide with the peaks of the growth rate. Aequorin measurements indicate that Ca$^{2+}$ concentrations at the tip can exceed 10 μM (Messerli and Robinson, 1997). Not only does the intracellular gradient oscillate during pulsatile growth but an extracellular Ca$^{2+}$ "influx" pulses with a phase lag of 149 degrees with respect to the growth pulses (Holdaway-Clarke et al., 1997). The term ‘influx’ appears in quotes because it is not known whether this Ca$^{2+}$ flux is really crossing the cell membrane. As this influx lags growth by such an extreme, it was proposed that it may be used for refilling intracellular stores through capacitative coupling or may enter Ca$^{2+}$ binding sites in the newly exposed cell wall (Holdaway-Clarke et al., 1997).

In addition to Ca$^{2+}$, the other external ions required for growth of lily pollen tubes are K$^+$ and H$^+$. Lily pollen tubes require a [K$^+$] of at least 0.01 mM and a pH of less than 6.5 (Weisenseel and Jaffe, 1976). We have shown that cytoplasmic pH pulses during pulsatile growth (Messerli and Robinson, 1998). Following each growth pulse, the cytoplasmic tip becomes more acidic, and each episode of acidification moves down the shaft of the tube as it dies out. The peak of the acidification lags the peak of the associated growth pulse by about 10 seconds and the pH may change by a full unit during these episodes. Unlike Ca$^{2+}$, the H$^+$ gradient disappears completely between pulses, and shorter tubes that are not yet growing in a pulsatile manner have no discernable pH gradients. There are also pulses of net ionic current that are approximately coincident with the cytoplasmic acidification, and K$^+$ appears to be a component of the current (Messerli and Robinson, 1998). This suggested the possibility that H$^+$ and K$^+$ were entering simultaneously. This led us to suggest the hypothesis that H$^+$ and K$^+$ enter the pollen tube tip simultaneously, perhaps via a H$^+$/K$^+$ symporter. Implicit in this hypothesis is the condition that the source of protons for cytoplasmic acidification is the extracellular medium. In order to test this idea we have used the SELF-Referencing Ion-Specific (SERIS) probe to measure the spatial and temporal fluxes of H$^+$ and K$^+$ near the surface of growing lily pollen tubes. In addition, we have used the Ca$^{2+}$ SERIS probe to confirm the surprising finding of Holdaway-Clarke et al. (1997) mentioned above. Knowledge of basic ion transport in this rapidly growing system is necessary for understanding the mechanisms controlling growth.

The SERIS probes are microelectrodes that have 1-3 μm diameter tips filled with an ionophore cocktail, each having a specific ionic selectivity. A concentration difference of the sensed ion across the cocktail produces a Nernstian potential difference between the inside and outside of the microelectrode, so an ionic concentration difference in the medium is detected as a voltage difference as the electrode is moved between two positions in the extracellular medium. Application of the equations derived from the laws governing diffusion allows the calculation of ionic fluxes from the measured concentration differences.

An important problem in the analysis of SERIS-measured flux data is the correlation of that data with the dynamic growth of the pollen tubes. The requirements of the electrophysiological measurements impose restrictions on the magnification and working distance of the microscope objective lens, with the result that the images of the tubes are typically not of high quality. This makes high-resolution analysis of growth from the video images difficult; in addition, manual analysis of the many hundreds of video frames is time consuming and tedious. Also, an inherent limitation of any manual analysis system is a spatial resolution of a single pixel. We have employed a newly developed computer vision system to track tip movement and thus determine growth rates of lily pollen tubes from digitized videotape. This system, described below, produces an automated evaluation of the image data with high efficiency and reproducibility. The high spatial sensitivity permitted us to analyze the video frames at the rate of 1 Hz. As a result, a complete characterization of the growth pulses at the sub-pixel scale was made possible, enabling us to measure growth with a spatiotemporal resolution of up to 1/10 pixel/second.

**MATERIALS AND METHODS**

*Lilium longiflorum* pollen grains were collected from the anthers of greenhouse grown flowers, dried for 2 days and stored at –20°C until use. For the experiments, a small amount of pollen was hydrated in modified Dickinson’s medium (in mM: 0.16 H$_3$BO$_3$, 0.127 Ca(NO$_3$)$_2$, 1.0 KNO$_3$, 5.0 MES, 292.0 sucrose) pH 5.5 with KOH. The pollen was then placed in a 25°C incubator, which causes the pollen to germinate within an hour of hydration. Hydrated pollen grains were then placed in 35 x 10 mm Falcon tissue culture plastic dishes which had been coated with 0.5 mg/ml poly-L-lysine in order to cause the tubes to adhere to the bottom of the dish (Messerli and Robinson, 1998). Experiments were performed at ambient temperature, 21-23°C.

**Flux measurements**

The self-referencing probe design has been described previously (Smith et al., 1994). Briefly an ionophore cocktail (H$^+$, Hydrogen Ionophore I - Cocktail B, K$^+$, Potassium Ionophore I - Cocktail B, Ca$^{2+}$, Calcium Ionophore Cocktail A, Fluka Chemical Co., Milwaukee, WI) was tip-loaded into a silanized microelectrode that had been backfilled with an ionic solution specific for the cocktail. The K$^+$ electrodes were back-filled with 100 mM KCl while the H$^+$ electrodes were back-filled with 100 mM KCl and 5 mM MES (pH 5.5 with KOH). The Ca$^{2+}$ electrodes were back-filled with 100 mM CaCl$_2$. A 3 M Na acetate bridge was used during H$^+$ and K$^+$ recordings while a 3 M KCl bridge was used during the Ca$^{2+}$ recordings. The K$^+$ electrode was calibrated by immersing it in 0.1, 1.0 and 10 mM KCl solutions. The logarithm of the concentration was plotted against the millivolt output to produce a linear curve, which was used to calculate the K$^+$ concentration of the medium and to calculate the small changes in K$^+$ concentration between electrode excursion points, 10 μm apart, which were then used to calculate flux. The probe was oscillated parallel to the plane of the microscope stage and along a radius to the artificial point source. The H$^+$ and Ca$^{2+}$ selective probes were calibrated in a similar manner using 10-fold concentration changes of H$^+$ and Ca$^{2+}$, respectively. During tube measurements the probe oscillated parallel to the plane of the microscope stage and perpendicular to the direction of tube growth in most cases.

Flux about a curved surface decays as the square of the distance from that surface. The self-referencing probe measured the ion concentration within no less than 1 μm of the membrane and then 10 μm further away. In order to determine the ion flux within 1 μm of the membrane we extrapolated the difference in ion concentration between these two points to determine the flux at the tube tip and grain (spheres) and the shaft of the tube (cylinder). The quantity of substance moving per unit time between surface 1 with radius $a$ and surface 2 with radius $b$ is equal to

$$4\pi D \frac{ab}{a-b} \frac{dC}{dc}$$

for a sphere, where $D$ is the diffusion coefficient of the ion and $dC$ is the measured concentration difference between the two surfaces.
(Crank, 1975). This value divided by the surface area of the tip, $2\pi r^2$, gives a close estimate of the flux at the cell surface. We assume, based on our measurements, that the pulsing flux is restricted to the hemispherical surface of the tip. In order to determine the steady H+ fluxes along the cylindrical portion of the tube, we use

$$J_a = \frac{2\pi D}{\ln b/a} \frac{dC}{dt}$$

(Henriksen et al., 1992) and divide by $2\pi$ to report on the flux per unit length. We used the simple relationship of

$$J_a = \frac{\mu b^2}{a^2}$$

to estimate the flux at the surface of the grain, where $J_a$ is the flux at the surface with radius $a$ and $J_b$ is the measured flux at the surface further away with radius $b$. Flux values are reported in the text as the surface calculated values unless otherwise stated as being the actual measured values. All graphs show the measured flux values. All numbers are given as the mean ± s.e.m. unless noted otherwise.

**Electrode efficiency**

The H+ and Ca2+ electrodes require ≤5 seconds to reach 90% of their maximum response while the K+ electrode requires <1 second. The slow response time of the electrodes compared with the more rapid 0.5 Hz oscillations means that the electrodes never fully measure the peak signal. The electrode efficiency was determined for each cocktail to determine the fraction of signal that was not detected due to the response time of the cocktails. The electrode efficiency for K+ was determined by taking measurements from a source electrode filled with 100 mM KCl and 1 mM LiCl in 1% agar in a bath of 100 mM LiCl and 1 mM KCl. A static measurement was taken near the source and then 10 μm further away from the source for approximately 15 seconds each. Then the probe was translated between these two points, 10 μm apart, and the difference between the two points was calculated by the SERIS software. The 15 second measurements allowed the probe to reach its maximum value at each point while the more rapid oscillation between the two points caused the probe to measure only a fraction of that value. A ratio of the concentration difference measured by the oscillating probe with the difference in concentration determined from the stationary measurements shows that the K+ electrode measures only 69% of the actual K+ gradient while the H+ and Ca2+ electrodes measure only 65% of the actual gradient for their respective ions.

**Correction for H+ buffering**

Extracellular H+ gradients will inevitably be influenced by buffering in the medium. The gradient is affected by the diffusion of both protonated and unprotonated species. A ratio to determine the amount of H+ moving as protonated buffer rather than as free H+ would be helpful to more accurately report the H+ gradient in buffered medium. A correction factor for this buffering capacity has been derived previously (Arif et al., 1995; Demarest and Morgan, 1995). Based on these equations we have calculated that the H+ buffer enhances the flux by an average factor of 19, for an average pH of 5.5, with 5 mM MES (pKa 6.1) with a diffusion coefficient of 0.66×10⁻⁵ cm² second⁻¹.

**Video measurements**

Video sequences were recorded on a Panasonic AG-6730 s-VHS videotape recorder (Secaucus, NJ) during SERIS recording, and then digitized at 1 frame per second with a resolution of 640×480 pixels by an InVideo PCI video card (Focus Enhancements, Sudbury, MA). From these 1-second digitized images we selected every third image. With this scheme, the position of the probe was near the tube and interfered with the image of the pollen tube on each fourth selected image. So instead of collecting the fourth image 3 seconds after the third image we selected the fourth image 1 second after the third image. This caused us to collect on average, 3 images, each one 3 seconds after its previous image with the fourth image occurring 1 second after its previous image. After image screening the frame rate of the digitized sequences ranged from 0.17-1 Hz (0.4 Hz average). These sequences were then exported as series of TIFF images to the tracking software.

There are sources of uncertainty in the temporal registration of the growth rate data and the ion flux data that must be mentioned. One source of temporal uncertainty arises from digitization of video. While the frames were collected every second, the beginning of digitization was never immediately at the beginning of a new time-stamped second of video. We estimate a lag of ≤0.5 second that would make the growth pulses appear to occur about 0.5 second earlier than they really did. Also, during SERIS data acquisition, a running average of 4 seconds was used to filter noise. A model of this type of averaging predicted that a flux lag of 1.5 seconds would occur but a direct empirical test of the probe using an artificial ion source produced a temporal lag of only 0.5 second. In combination, these sources of uncertainty shift the ion fluxes about 1 second later with respect to the growth rates when compared to their actual relationship. This uncertainty was not taken into account when determining the phase relationships between the fluxes and the growth pulses.

**RESULTS**

**Steady and pulsatile H+ and K+ fluxes**

Ionic fluxes of H+ and K+ were measured around the grains (point A, inset to Fig. 1), and growing tips of lily pollen tubes (points A and B, inset to Fig. 1). In shorter tubes, which were growing in a steady manner, we could not detect any K+ fluxes but could measure steady H+ flux. H+ efflux occurred at a steady, average rate of 21.5±6.2 pmol cm⁻² second⁻¹ (n=3) from the grain. The average steady influx at the tip surface was 6.8±0.8 pmol cm⁻² second⁻¹ and increased to 17.4±2.2 pmol cm⁻² second⁻¹ per unit length at 19.4±1.7 μm behind the tip (point B, inset to Fig. 1) before declining to nearly immeasurable levels within 40 μm behind the tip (n=5). Surface flux at a region between 50 and 100 μm from the grain (point C, inset to Fig. 1) showed a very small influx, averaging 3.1±0.4 pmol cm⁻² second⁻¹ per unit length (n=3).

The average medium concentration of H+ was measured to be 3.9±0.9 μM and 3.6±0.3 mM, respectively. The background [H+] along with a H+ electrode noise of ±5 μV resulted in a flux noise of ±2.1 pmol cm⁻² second⁻¹. However, the higher background [K+] along with a higher electrode noise, ±10 μV, resulted in a flux noise of ±43.1 pmol cm⁻² second⁻¹. Based on the background concentration of the ions and electrode noise, H+ fluxes one order of magnitude lower than the K+ fluxes could be measured. This may explain why we could detect basal H+ fluxes but not basal K+ fluxes.

Flux measurements at the tip, acquired during pulsatile growth, showed pulsatile influx of both H+ and K+. The average measured flux at the peaks of the H+ pulses was 160±34 pmol cm⁻² second⁻¹ (158 pulses from 7 tubes). H+ pulses were detected in all tubes that were growing in a pulsatile manner. The average measured flux at the peaks of the K+ pulses was 283±54 pmol cm⁻² second⁻¹ (127 pulses from 8 tubes). K+ flux was not detected in 2 tubes that were growing in a pulsatile manner. Due to the greater sensitivity of the H+ probe, we measured 29 H+ pulses with amplitudes that would have been immeasurable with the K+ probe. With these measurements we determined that the
flux at the surface of the membrane was 489±81 and 688±144 pmol cm$^{-2}$ second$^{-1}$ for H$^+$ and K$^+$, respectively. We found that the tubes that had measurable K$^+$ fluxes had an average tube diameter of 18.5±0.6 μm while the H$^+$ tubes had an average diameter of 16.7±0.8 μm. We performed regression analysis of flux amplitude with tube diameter and found that larger flux amplitudes do not correspond with larger tube diameters for H$^+$ ($y=26.5x-283.1$, $P>0.1$) but do correspond for K$^+$ ($y=98.0x-1531.9$, $P<0.05$) measured tubes. (H$^+$: the slope of the fitted regression line is zero). With the best fit line for the K$^+$ points we see that tubes with a diameter ≤16.1 μm have a predicted K$^+$ flux which is less than the K$^+$ background noise of 43.1 pmol cm$^{-2}$ second$^{-1}$. The two tubes that were growing in a pulsatile manner without measurable K$^+$ fluxes were 15.8 and 16.0 μm in diameter, which may explain why we could not detect pulsing K$^+$ fluxes in these tubes. The smallest diameter tube from which we measured K$^+$ fluxes was 16.7 μm.

During some pulsatile H$^+$ flux measurements, we allowed the tube to grow past the probe while it continued to measure fluxes. Fig. 1 shows two recordings of a tube with high amplitude H$^+$ influx as it grew past the probe’s measuring position. The probe was oscillating perpendicular to the direction of growth and did not change during collection of these data. Tip measurements were taken within 5 μm of the leading edge of growth. The SERIS probe appears in the upper right side of the images. Numbers in the upper left of the images, display the frames of this movie that were used. The second of these two numbers for each image is the number of the shown frame. The boxes and points display the selected region of interest and point of interest, respectively. The blue points indicate the point of interest from the preceding image while the red points indicate the point of interest of the current frame. The last image shows an accumulation of all of the points of interest for 52 frames of this movie. The region and point of interest were selected in the first frame of each movie and the computer tracker followed these for the rest of the frames of the movie. The vector difference of the pixel coordinates of the point of interest was used to calculate growth rates for the tubes. Frame 4>5 shows a large rotational component of growth (see text).
tube’s tip. Measurements behind the tip are continuous recordings and the distances on the graph show the position of the probe with respect to the tip of the tube. It can be seen that the pulsing H⁺ influx could be detected behind the tip with a stronger baseline influx occurring as the peaks of the H⁺ pulses decreased. Similar to the steady tracking tubes we could not detect efflux of H⁺ within 50 μm behind the tips of the tubes. This suggests that the large amount of intracellular H⁺ is not pumped back out of the cytoplasm near the tip of the tube.

**Tracking growth**

In order to produce growth measurements with higher resolution and greater repeatability we employed the use of tracking software. A technical description of the tracking software appears in the Appendix to this paper. The typical tracking scenario is illustrated by Fig. 2. This shows the first five tracking steps of a sequence with 52 frames. The results from tracking step \(i \rightarrow i+1\) are in each case overlaid to frame \(i+1\). The regions of interest (ROIs) are painted as blue windows. The same window transformed according to the estimated transition model is shown in red. Note both the translation and the rotation components are necessary to describe the evolution of the pollen tube tip. In all the tracking experiments the ROI for the very first step 1\(\rightarrow\)2 had to be selected manually. For all subsequent steps \(i \rightarrow i+1\) the position of the ROI was derived from the former steps \(j \rightarrow j+1\), \(\forall j=2...i\). Also, in the first frame, the operator had to initialize a point of interest (POI) located on the leading edge of the pollen tip (shown as a blue point in the picture for step 1\(\rightarrow\)2).

Based on the estimated transition model from frame 1 to 2 the POI was relocated in frame 2. The position is shown as a red overlay to the picture for step 1\(\rightarrow\)2. In the picture for step 2\(\rightarrow\)3 this same point is drawn in blue. Its updated position after the step 2\(\rightarrow\)3 is plotted in red. The tracking of the POI was continued accordingly throughout the full sequence. The POI’s trace over all 52 frames is overlaid to frame 52 in the lower right picture of Fig. 2. The growth rates were then calculated as the derivative of the trace with respect to time. For the trace shown in Fig. 2 notice the visible gap in the beginning of the path which corresponds to the step 4\(\rightarrow\)5. It is mainly the rotation component of the transition model that causes this rather large step. An abrupt change in growth direction was detected at this moment which was properly described by the tracker. As claimed in the Introduction, the sensitivity of the tracker reaches a level of about 1/10 of a pixel. Fig. 3A shows estimated growth rates over 163 frames sampled at 0.2 Hz on average. This tube was chosen as it was growing especially slowly, with growth pulses occurring at 0.0052 Hz, 3 minutes 11 seconds between pulses. The bold line depicts the magnitude in growth while the fine line displays the direction of growth, expressed as the angle between the horizontal axis of the image coordinate system and the growth vector. The directional data varies in a range ±45°. As expected from the biological background of the experiment a steady growth in one prominent direction was observed. There was no step measured in the opposite direction that would cause a break of about ±180° in the directional data. Such an opposite step could appear either because of actual retraction of the tube or due to measurement artifacts. Yet, most of the measured displacements were smaller than 1/2 pixel, Fig. 3B. The agreement in direction between lower and higher growth rates qualitatively confirms the statistical significance of even the smallest displacement values obtained.

**H⁺ and K⁺ phase analysis**

With computer vision we were able to perform growth rate measurements at 1 second resolution. However, due to the 2.5 second average resolution that the flux data was collected we chose to use a 3-second measurement interval (average 2.5 seconds). This is adequate to describe the 42 second average period growth pulses of the tubes. Fig. 4A and B show growth pulses and H⁺ fluxes from two different tubes. The H⁺ pulses showed two classes of pulsing amplitudes, low amplitude pulses intermixed with high amplitude pulses, Fig. 4A, and primarily high amplitude pulses, Fig. 4B. The magnitude of the peaks of the growth pulses are directly proportional to the peaks of the subsequent H⁺ pulses (regression: \(P<0.0001\)) while the magnitudes of both the growth pulses and H⁺ pulses are inversely proportional to the subsequent drop in growth rate (regression) \(P<0.001\) and \(P<0.0001\), respectively. Not only are the high amplitude H⁺ pulses followed by a drop in growth rate but the onset of the next growth pulse is delayed following very high amplitude H⁺ fluxes. Examples of this have been marked

![Fig. 3](image-url) (A) Growth rate in pixel(s)/frame and growth direction of the image set shown in Fig. 2 spanning the full sequence of 163 frames. The growth rate plot is displayed as a bold line while the growth direction plot is displayed as a thin line. The growth direction varies inversely proportional to the subsequent drop in growth rate (regression) \(P<0.001\) and \(P<0.0001\), respectively. Not only are the high amplitude H⁺ pulses followed by a drop in growth rate but the onset of the next growth pulse is delayed following very high amplitude H⁺ fluxes. Examples of this have been marked.
on Fig. 4A and B with arrows. The peaks of growth pulses and the amplitudes of the H⁺ fluxes are proportional to the amount of time before the peak of the following growth pulse (regression $P<0.001$ for both). Fig. 5A and B show growth pulses and K⁺ fluxes from two different tubes. The K⁺ pulse amplitudes are also proportional to the preceding growth pulses (regression: $P<0.001$). The peaks of the amplitudes of the K⁺ fluxes did not show as much variability as the H⁺ fluxes. However, K⁺ pulses with the same amplitude as the smaller H⁺ pulses would not be detected with the K⁺ probe. Consistent with this is a growth pulse in Fig. 5B, marked with an arrow, which has no detectable K⁺ pulse above the noise level. We observed 5 cases such as this. While there does appear to be a K⁺ pulse that occurs with that growth pulse there is an equal amplitude K⁺ pulse which occurs at about 90 seconds on this recording which does not contain a growth pulse. The amplitudes of both of these pulses are less than the noise level of the probe.

We determined the relationship of the ion pulses to the growth pulses by performing correlation analysis (Holdaway-Clarke et al., 1997; Messerli and Robinson, 1998) between the slopes of the ion flux data and growth rate data. We found that the H⁺ pulses most strongly correlate with growth when they lag the growth pulses by 11.4 seconds (158 pulses from 7 tubes). The K⁺ pulses most strongly correlate with growth when they lag the growth pulses by 13.8 seconds (91 pulses from 6 tubes). We also measured the average period of the growth pulses for the tubes and found that the H⁺ measured tubes had an average period of 41.8±4.0 seconds while the K⁺ tubes had an average period of 52.8±5.3 seconds. When the ion flux lag is compared to the respective average period of each tube, it is found that the phase lags of the H⁺ and K⁺ pulses are 103±9 and 100±11 degrees; these lags are not statistically different from each other ($P>0.5$). Fig. 6 shows these correlation plots of the ion fluxes and growth rates. These plots encompass a full period before 0 degrees to show that the ion fluxes more closely correlate when lagging the preceding
growth pulse rather than leading the subsequent growth pulse. This difference in average period between the H⁺ and K⁺ measured tubes appears to correspond with the diameters of the tubes. A regression analysis indicates that large tube diameters correspond with long periods between growth pulses ($P<0.002$, $n=19$). This shows that larger diameter tubes have lower frequency pulses and would explain the periodic difference between the H⁺ and K⁺ measured tubes. Specifically, K⁺ pulses could only be detected in larger diameter tubes and these larger tubes had lower than average pulse frequencies.

**Membrane potential**

The membrane potential, $V_M$, of lily pollen tubes was measured between 50 and 100 $\mu$m behind the growing tip. Fine-tipped electrodes (30 MΩ) were pulled to minimize damage upon impalement. Any damage that occurred during impalement would serve to reduce the magnitude of the membrane potential. The measured membrane potential was $-102\pm10$ mV (s.d.) ($n=24$) in 3 mM K⁺. We compared this value to the calculated potassium equilibrium potential, $E_K$, assuming 124 mM [K⁺] as estimated by Weisenseel and Jaffe (1976). We found that the $E_K$ in 3 mM K⁺ is about 10 mV more positive, $-94$ mV, than the $V_M$. The membrane potential is sufficiently negative to drive K⁺ influx passively through ion channels although this is a fairly small driving force for K⁺ and may not be the only mechanism for K⁺ entry. However, it should be realized that the membrane potential is, if anything, underestimated so the difference between $V_M$ and $E_K$ may be larger than our current estimate.

**Ca²⁺ fluxes and phase analysis**

Ca²⁺ fluxes were measured primarily at the tips of longer tubes growing in a pulsatile manner. Ca²⁺ flux was directed toward the tip of the tube and occurred in a pulsatile manner with the growth pulses. The measured background concentration of Ca²⁺, $0.15\pm0.01$ mM, along with the electrode noise, ±5 μV, was used to determine a flux noise level of 0.51 pmol cm⁻² second⁻¹. Fig. 7A shows a tube during steady growth with a relatively steady tip-measured Ca²⁺ flux while Fig. 7B shows that same tube 63 minutes later, during pulsatile growth. It can be seen that the

![Graph A](image1)

![Graph B](image2)

**Fig. 5.** (A,B) Growth rates and tip-measured K⁺ influx from two different pollen tubes. K⁺ influx lags growth by 100±11 degrees. (A) K⁺ influx was measured only in larger diameter tubes, 8 out of 10, and showed lower pulsing frequencies. (B) Recording from a tube with a growth pulse that is missing a corresponding K⁺ pulse. If a ratio of 1, H⁺:K⁺, exists, there will be K⁺ pulses that have amplitudes below the noise level of the K⁺ probe.
from 6 tubes), which is not significantly different from the H+ average growth pulse period of 39.5±2.9 seconds (188 pulses found that the Ca2+ pulses lag the growth pulses by 13.2 seconds. With the image tracker and correlation analysis, we -flux of 22.1±3.8 pmol cm-2 second-1 of 7.5±1.6 pmol cm-2 second-1 to the magnitude of the Ca2+ pulse (regression, P<0.001, for all 7 tubes). With the image tracker and correlation analysis, we found that the Ca2+ pulses lag the growth pulses by 13.2 seconds. This corresponds to a phase lag of 123±9 degrees from an average growth pulse period of 39.5±2.9 seconds (188 pulses from 6 tubes), which is not significantly different from the H+ flux lag (P>0.1) or the K+ flux lag (P>0.1). The Ca2+ phase lag is shown in Fig. 6 along with the H+ and K+ correlation curves. We removed the tube with the 3 minutes 11 seconds period from the phase analysis as we have never seen another tube with such low frequency pulses and consider it abnormal. The Ca2+ pulses lagged the growth pulses of this tube by 20 seconds, a phase lag of 37.9 degrees.

**DISCUSSION**

**H+ fluxes**

Our results offer direct confirmation of our previous inference that the measured intracellular H+ pulses were due to the entry of H+ from the extracellular space (Messerli and Robinson, 1998). We have measured two types of extracellular pulsing H+ fluxes with the SERIS probe. Namely, there are tubes that show constantly large pulses of H+ entry and there are tubes that show large pulses intermixed with smaller pulses. The high amplitude H+ fluxes follow high peak growth rates and these both precede more dramatic decreases in growth rate and longer times before the peak of the next growth pulse. We conclude that the cytoplasmic H+ pulses are the result of pulsing H+ influx that has been measured with the SERIS probe. Not only do the extracellular H+ fluxes and the H+ pulses occur with the same time lag with respect to growth (Mann-Whitney P>0.2) but they show the same proportional amplitude variation and the large amplitudes of the measured H+ fluxes can cause the dramatic intracellular [H+] changes. The average amount of H+ that enters the tips over a 30 second pulse duration is 33.7 pmol, assuming the measured influx occurs over the tip of the tube, which is modeled as a hemisphere with a radius of curvature equal to half the tube diameter. This amount of H+ distributed over the cytoplasmic volume of the distal 10 μm corresponds to a [H+], of 20.5 mM and over the distal 20 μm corresponds to an increase of 8.5 mM. These are crude estimates as the [H+], is not spread equally over these volumes but exists as a tip-high gradient. With calculated H+ concentrations in the millimolar range and with peak intracellular measurements of 1 μM, the cell appears to posses a buffering capacity in the tens of millimolar. This value is well within reported cytosolic H+ buffering ranges (Grabov and Blatt, 1997; Plieth et al., 1997) giving us confidence that the concentration of intracellular H+ indicator (less than 50 μM) used to measure the intracellular H+ pulses (Messerli and Robinson, 1998) had negligible effects on the already high cytoplasmic H+ buffering capacity.

**K+ fluxes**

We have previously reported that pulsatile K+ influx may lag growth pulses (Messerli and Robinson, 1998). With the SERIS system, we show this directly. The K+ and H+ pulses are remarkably similar with respect to their timing, average peak amplitude and proportional relationship to the preceding growth pulse. The close correspondence in timing and magnitude of the H+ and K+ influx pulses suggests the possibility that a H+/K+ cotransporter is responsible for the measured fluxes. The H+/K+ cotransporter is thought to aid K+ uptake under low extracellular K+ conditions (Maathuis and Sanders, 1994). Even if H+/K+ cotransport occurs during the growth pulses, it need not be the mode of K+ influx during slower rates of growth. Our measurements of the membrane potential suggest that it is sufficiently negative to drive K+ influx. Perhaps during the higher rates of growth, K+ influx cannot keep up with the increase in volume and the cell uses the steep electrochemical H+ gradient to drive more rapid accumulation of K+ by an active mechanism. What role might these K+ pulses play during growth? One possibility is that the K+ influx acts to restore either the total ionic concentration and/or osmotic concentration after the increase in cell volume. We determined the influence of the K+ influx on the intracellular K+ concentration and osmotic pressure for the new tube volume. Integrating over a growth pulse lasting 30 seconds with an average amplitude of 688 pmol cm-2 second-1 and an average growth rate of 0.22 μm/second, we find that the total amount of K+ influx accumulates to 31.3 mM in the new volume of tube. This contributes to 25% of the necessary K+ concentration, 124 mM (Weisenseel and Jaffe, 1976), for the new tube volume and to 37% of the average hydrostatic pressure, 0.209 MPa (Benkert et al., 1997), because it is the difference in osmolarity between the cytoplasm and medium that generates turgor pressure. It is clear that the K+ flux has

![Fig. 6. Correlation analysis of the pulsing ion fluxes and the pulsing growth rates. The H+, K+, and Ca2+ fluxes lagged growth by 11.4, 13.8 and 13.2 seconds, respectively.](image-url)
a larger effect on the osmotic concentration of the cell than on the total final K⁺ concentration. We also think that the cell is more responsive to small changes in turgor pressure rather than small intracellular K⁺ changes and therefore suggest that the K⁺ influx is helping the cell to rapidly recover turgor pressure for the increased volume of the tube.

Fig. 7. (A-C). Growth rates and tip-measured Ca²⁺ influx acquired from two pollen tubes. (A) At early stages of growth, tubes grow in a steady fashion and show a relatively steady tip-measured Ca²⁺ flux. (B) Measurements from the same tube as in A but acquired 63 minutes later. The tube shows pulsatile growth and pulsatile influx of Ca²⁺. The average growth rate of the tube is greater but the average Ca²⁺ influx remains about the same because the pulsing Ca²⁺ magnitudes oscillate about the average Ca²⁺ level shown in A. (C) Measurements acquired from a different tube that shows faster growth and larger amplitude Ca²⁺ fluxes. Ca²⁺ influx lags growth by 123±9 degrees.
Ca\textsuperscript{2+} fluxes

We have confirmed the surprising extracellular lag of Ca\textsuperscript{2+} influx compared to growth and the intracellular Ca\textsuperscript{2+} changes reported by Holdaway-Clarke et al. (1997). We measured an average temporal lag of 13.2 seconds which corresponds to a phase lag of 123±9 degrees. This is slightly less than the 149±4 degree lag reported by Holdaway-Clarke et al. (1997) but still significantly lags intracellular Ca\textsuperscript{2+} pulses, which occur nearly in phase with growth pulses (Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). Our flux amplitude measurements are nearly identical to those reported by Holdaway-Clarke et al. (1997). However, we have introduced a correction for the electrode efficiency coefficient and have used the geometry of the cell to calculate the flux that occurs at the cell surface to more closely describe the amplitudes of the pulses. Like the H\textsuperscript{+} and K\textsuperscript{+} fluxes, the amplitudes of the Ca\textsuperscript{2+} fluxes are proportional to the magnitude of the preceding growth pulse. However, the Ca\textsuperscript{2+} fluxes are an order of magnitude lower than the H\textsuperscript{+} and K\textsuperscript{+} fluxes. The observation that the Ca\textsuperscript{2+} flux oscillates about the steady growth baseline influx is reminiscent of our observations that during the intracellular Ca\textsuperscript{2+} pulses, the [Ca\textsuperscript{2+}]\textsubscript{i} oscillates about the steady [Ca\textsuperscript{2+}]\textsubscript{i} which exists during steady growth (Fig. 1A, Messerli and Robinson, 1997).

The fact that the extracellular Ca\textsuperscript{2+} fluxes lag the intracellular pulses by about 1/3 of a cycle is quite puzzling. Holdaway-Clarke et al. (1997) suggested two possible explanations of this matter. First, it may be that the probe-measured fluxes are not entering the cell at all but rather are binding to newly exposed anionic sites in the pectin layer of the cell wall. Alternatively, the Ca\textsuperscript{2+} may be entering the cell to refill depleted intracellular stores. In favor of the former alternative is the correspondence between the number of new anionic sites created and the total Ca\textsuperscript{2+} flux. However, it offers no explanation of what the source of the Ca\textsuperscript{2+} is for the observed intracellular Ca\textsuperscript{2+} pulses. If the source of Ca\textsuperscript{2+} is intracellular stores and not extracellular influx, then when are those stores refilled? The original model of

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Fig. 8. Comparison of growth rate data collected with the computer vision tracker and the VIA-100 system. A1 and B1 are plots of the data collected from computer vision (2.5 seconds resolution) and the VIA system (5 seconds resolution), respectively. The ‘stars’ in B1 correspond to points that are off scale. A2 and B2 show the results of 3-point smoothing of A1 and B1. It can be seen in B1 that the measurements are limited to the size of a single pixel as the data can be placed in 6 equally spaced rows, indicated by the arrows. The smoothing of this single pixel resolution data will show the general trends of the data and reduce noise, B2. The lower spatial resolution of the VIA system caused us to collect data points every 5 seconds. The low signal to noise measuring ability caused us to miss two growth pulses with the VIA system, indicated by arrows 1 and 3. B2. Also the single data point, at arrow 2, indicating the low point of growth cannot by itself be distinguished as a low point of growth or merely noise of the measuring system. C shows the corresponding Ca\textsuperscript{2+} influx of this tube. The growth pulses of the smoothed computer vision data, A2, are easily matched with the corresponding Ca\textsuperscript{2+} fluxes shown in C.
capacitative calcium entry posited that intracellular \( \text{Ca}^{2+} \) stores were refilled directly from the extracellular medium, but that idea has been abandoned and it is now thought that the \( \text{Ca}^{2+} \) that enters to refill the stores must cross at least some span of cytoplasm (Parekh and Penner, 1997). If this is the case, why is no indication of this seen in the \( \text{Ca}^{2+} \) imaging records? We are forced to conclude that some pieces of this puzzle are missing. One important missing piece is any information about the nature of the ion channels that are involved in regulating the entry of \( \text{Ca}^{2+} \) into the pollen tube tip.

**Strength of computer vision**

There is a tug-of-war that occurs between spatial and temporal resolution when performing measurements on growing cells. Enough time must elapse in order for a measurable amount of growth to occur, but if too much time is allowed to elapse between measurements then growth information can be lost. On most current digital measuring devices this conflict can be understood in terms of a ratio of the growth rate of the cell (distance/second) to the spatial resolution of the image (distance/pixel). This ratio, in pixels/second, can be enhanced with magnification, allowing the same amount of growth to occur over a larger number of pixels, but this can only be useful to a certain extent before empty magnification and blurring occur. Additionally, certain procedures and/or certain culture chambers must be used with low magnification objectives. An alternative is to go in the other direction and make the pixels effectively smaller i.e. measure in the subpixel domain. To show the spatial/temporal advantage of subpixel measurements we have directly compared the growth rate of a pollen tube measured with computer vision and that measured with the VIA-100 system from Boeckler (Tucson, AZ) which we have previously used (Messeri and Robinson, 1998). A plot of the computer tracking measurements are shown in Fig. 8, A1 while a VIA measurement is shown in Fig. 8, B1. For this magnification of tube we thought that it was best to acquire measurements every 5 seconds. In B1 the blocks can be seen to line up neatly into rows showing the actual unity pixel measurements while the points in A1 blend together. The VIA data clearly shows periodic pulses in some instances but shows ambiguous pulses in other instances. In order to find the general trends in growth for the VIA system we perform 3-point averaging so that each point is really the average of itself and the nearest point on each side. These numbers are shown in Fig. 8, B2. For comparison we did this same averaging for the computer tracked data and show it in A2. The general trends of the VIA data show pulsing growth except at arrows 1 and 3 where two growth pulses are missing. We can clearly see these two pulses in the computer vision data, A2. We have reason to believe that there were pulses at these points because we can see the corresponding \( \text{Ca}^{2+} \) pulses for these growth pulses in Fig. 8, C. Another feature to note is that at arrow 2 in Fig. 8, B2, there is a single point indicating the low point of a growth pulse. It is difficult to determine if this single point is a real event or a noise artifact. With higher resolution measurements we can acquire more data points between pulses to really show the bases and peaks of the growth pulses.

**Conclusions and future focus**

Early experiments on lily pollen indicated K\(^+\) influx over the whole tube and \( \text{H}^+ \) efflux from the grain (Weisenseel and Jaffe, 1976). Also \( \text{H}^+ \)-ATPase localization with monoclonal antibody labeling showed an abundance of these pumps at the grain, which are absent or sparsely distributed on the tube (Obermeyer et al., 1992). We have now performed regional measurements of global \( \text{H}^+ \) and K\(^+\) transport and we confirm that \( \text{H}^+ \) efflux does occur at the grain. We measured small \( \text{H}^+ \) influx along the tube 50-100 \( \mu \text{m} \) away from the grain and then higher basal influx over the 40 \( \mu \text{m} \) behind the tip with the strongest steady influx occurring about 20 \( \mu \text{m} \) behind the tip. Two measurements from a strongly pulsing tube that was allowed to grow past the probe showed \( \text{H}^+ \) influx as far as 50 \( \mu \text{m} \) behind the growing tip. A question that arises from this information is where does all the \( \text{H}^+ \) go after such a large increase in the intracellular \( \text{H}^+ \) concentration. Large amounts of \( \text{H}^+ \) would surely be detected if it were pumped back out near the tip. We think that the \( \text{H}^+ \) is buffered by cytoplasmic organelles and then transported back to the grain via cytoplasmic streaming, where it is then pumped out. Evidence for this comes only from the measured fluxes of \( \text{H}^+ \), the tube being a site of measured influx and the grain being the site of efflux. We could not measure basal K\(^+\) influx against the high background K\(^+\) concentration but we have been able to measure tip-restricted K\(^+\) pulses. This indicates that the \( \text{H}^+ \) fluxes, K\(^+\) transport is also polarized within this cell. With polarized, positively charged influx, how is electroneutrality maintained? Is the efflux of positive charge at the grain sufficient to account for the large amount of positive charge influx at the tip? The total current flow for \( \text{H}^+ \), K\(^+\) and \( \text{Ca}^{2+} \) across the hemispherical tip region (average tube diameter 17 \( \mu \text{m} \)) sums to 0.55 nA. The total current leaving the grain in the form of \( \text{H}^+ \) efflux, 21.5 pmol cm\(^{-2}\) second\(^{-1}\) (average grain diameter 90 \( \mu \text{m} \)), is 0.53 nA. This means that enough positive charge leaves the grain to balance the entry of positive charge in the tube.

There have now been 5 dynamic events that have been shown to occur in association with pulsatile growth: (1) rise in the tip-high cytosolic \( \text{Ca}^{2+} \) gradient, (2) creation of a cytosolic, tip-high gradient of \( \text{H}^+ \), (3) \( \text{H}^+ \) influx, (4) K\(^+\) influx and (5) \( \text{Ca}^{2+} \) influx. With respect to growth pulses, the intracellular \( \text{Ca}^{2+} \) concentration changes nearly in phase with the growth pulses (Holdaway-Clarke et al., 1997; Messeri and Robinson, 1997). While Holdaway-Clarke et al. (1997) show that changes in the intracellular \( \text{Ca}^{2+} \) concentration slightly lead the changes in growth rate our data suggest that it slightly lags growth (unpublished results). This relationship is important for clarifying the relationship between the \( \text{Ca}^{2+} \) gradient and tip growth. Does a rise in the intracellular \( \text{Ca}^{2+} \) gradient occur first to promote tip growth or does a small amount of turgor-generated stretch occur to promote the rise in the intracellular \( \text{Ca}^{2+} \) gradient that promotes tip growth? These questions can be resolved through experiments that employ enhanced spatial and temporal measurements of tip-growth and the intracellular \( \text{Ca}^{2+} \) gradient. The second and third events we have measured are the intracellular changes in \( \text{H}^+ \) concentration and the \( \text{H}^+ \) influx from the medium. The intracellular \( \text{H}^+ \) pulses appear to be the result of extracellular \( \text{H}^+ \) influx. It may be that the rise in intracellular \( \text{H}^+ \) interferes with \( \text{Ca}^{2+} \) signaling and other cytosolic events such as actin polymerization; however, we do not have direct proof of this inhibitory role on growth and therefore plan to test this hypothesis further. The cytosolic \( \text{H}^+ \) pulses may also be the result of the activation of a \( \text{H}^+ \) transporter that drives K\(^+\) influx. Alternatively, the \( \text{H}^+ \) pulses may be the result of transport using the transmembrane electrochemical \( \text{H}^+ \) gradient to drive import of some other molecule such as sucrose or nitrate. Thirdly, we
have measured pulsing K⁺ influx that occurs with the same phase shift and similar magnitude as the H⁺ fluxes. While we suspect that a H⁺/K⁺ cotransporter may be involved with these pulses, we cannot rule out the possibility that an increase in K⁺ permeability occurs to cause the pulses. We think that the trigger for K⁺ influx is a drop in turgor and therefore we predict that a small drop in turgor pressure would occur after each growth pulse. However, small changes in turgor have not been measured in these tubes (Benkert et al., 1997). Perhaps this measurement technique is not sensitive enough to measure such changes. Finally, the extracellular Ca²⁺ influx lags the growth pulses by between 123 degrees (our data) and 149 degrees (Holdaway-Clarke et al., 1997). The Ca²⁺ influx may simply bind to anionic sites in the new cell wall. If this is the case, then why can’t the intracellular Ca²⁺ influx associated with cytoplasmic Ca²⁺ pulses be measured? The intracellular Ca²⁺ influx is thought to occur through tip-activated mechanosensitive channels (Malhó et al., 1995; Pierson et al., 1994). This model may be wrong and the rise in intracellular Ca²⁺ may be solely from intracellular stores. If the intracellular Ca²⁺ gradient is the result of release from intracellular stores and since no mechanism of refilling has clearly been found, then perhaps the Ca²⁺ released from intracellular stores is completely recycled after each release.

With extracellular measurements we have measured fluxes of H⁺, K⁺ and Ca²⁺. However, we do not think that these measurements account for all of the membrane ionic traffic. When we compare the current pulses, measured with the vibrating probe (Messerli and Robinson, 1998; Weisenseel et al., 1975) and the combined flux of these three ions, we find a large discrepancy. The cumulative average peak flux of these ions at the membrane is 1215 pmol cm⁻² second⁻¹ while the membrane calculated flux for the vibrating probe, assuming the charge is carried by a monovalent cation, is 64.4 pmol cm⁻² second⁻¹, 1/20 of the total expected flux. Since the current probe only measures net ionic movement, it cannot distinguish between influx of a positively charge ion and efflux of a negatively charged ion. Tip-restricted influx of negative charge or efflux of positive charge during the pulsing ion fluxes will reduce the net current influx measured by the current probe. Since the movement must occur at the same time as the H⁺, K⁺ and Ca²⁺ pulses and as no other positively charged ions exist in high concentrations within the cell, a negatively charged molecule may be entering the cell, for example, nitrate. It should be pointed out that the magnitude of the current flux, assuming influx of a divalent ion, 32.2 pmol cm⁻² second⁻¹, is nearly the same as the Ca²⁺ influx, 38.4 pmol cm⁻² second⁻¹. The current influx may simply be transmembrane Ca²⁺ influx and the H⁺ and K⁺ influxes may be involved in the electroneutral transport of other ions. The resolution of these issues must await the development of new techniques and their application to the physiology of pollen tube tip growth.

**APPENDIX**

**Computer vision software**

The central task in visual tracking is to solve a correspondence problem between subsequent frames of a movie, i.e. a set of features in the image of the target object has to be localized in the frame captured at time \( t \) and to be refound in the next frame taken after a time step \( dt \). What makes the finding of correspondence difficult is the potential variability in the images of the target over time. This variability arises from three principle sources: variation in object pose or shape, variation in image contrast, and interference with images of other objects in the scene. Correspondence can be searched for on either the level of the image signal or on a symbolic level. When tracking symbols, features such as landmarks, lines, or edges are independently extracted from both frames at \( t \) and \( t+dt \) and then brought to correspondence based on a search algorithm. Alternatively, signal-based tracking methods rely on solving the correspondence for each individual intensity sample within a region of interest (ROI). Symbol-based tracking methods have advantages in terms of robustness against the three above-mentioned sources of image variability. In most cases, the features of a target object are preserved or can be easily restored despite the changes in object pose, shape, or image contrast. However, the methods bear disadvantages in terms of sensitivity in detecting small displacements and, therefore, in terms of spatial resolution. This originates from splitting the algorithms in an initial procedure for feature detection with subsequent feature tracking. Inaccuracies acquired in the individual processing steps often add up in an unfavorable manner. Since growth rates had to be quantified with sub-pixel resolution a signal-based method was chosen for this work.

Briefly, for every intensity sample \( I_{i,j}(\xi,t) \) within an ROI of the frame acquired at time \( t \), the corresponding sample \( I_{i,j}(\xi,t+dt) \) is searched for in the frame captured at \( t+dt \). During the period \( dt \) brightness of a sample may change such that:

\[
I_{i,j}(\xi,t) = I_{i,j}(\xi,t+dt) + dl_{i,j},
\]

where

\[
dl_{i,j} = b + c I_{i,j}(\xi,t).
\]

The parameter \( b \) denotes the additive change in brightness and controls a model of linear contrast change within the ROI. Both \( b \) and \( c \) were estimated by intensity histogram equalization (Jain, 1989) between the ROI at \( t \) and its correspondent at \( t+dt \). The locations \( x \) and \( \xi \) of an intensity sample \( I_{i,j}(\xi,t) \) and its respective correspondent \( I_{i,j}(\xi,t+dt) \) are connected by a geometric transition model. The formulation of the transition model varies with the application. For tracking the leading edge of a pollen tube tip it generally sufficed to include translation and rotation, i.e. \( \xi = d + R(\Phi) \cdot x \). The vector \( d \) describes the tip displacement from one frame to the next while \( \Phi \) denotes the change in orientation of the leading edge. The change in orientation is folded into the transition model by introducing an orthonormal matrix \( R(\Phi) \) depending on \( \Phi \). Estimates for these parameters were found by:

\[
[dl, d\Phi] = \arg \min \int_{\text{ROI}} (I_{i,j}(x,t) - (I_{i,j}(x,t+dt) + dI_{i,j}))^2 \, dx.
\]

In words, displacement and change in orientation of the pollen tube tips were obtained by minimizing the squared sum of intensity differences (SSD) between all samples within an ROI covering the leading edge at time \( t \) and their correspondents at time \( t+dt \). Various mathematical solutions to this type of SSD minimization have been described in the literature (Danuser and Mazza, 1996; Davis and Freeman, 1998; Hager and Belhumeur, 1998). Importantly, the problem always has an analytical, though iterative, solution. As a consequence, SSD minimization can be achieved with only a few iterations, almost independently of the complexity of the geometric transition model. The solution applied to pollen tube tracking worked

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along the lines of Danuser and Mazza (1996). Compared to the other methods cited above this technique provides better accuracy and a larger area of convergence. It is, however, considerably slower because of the high computational costs. Therefore, in the case of real-time tracking one of the other minimization techniques might be advantageous.

Although the operator chooses a rectangular window as an ROI only the intensity samples along the leading edge of the tube have significant impact on the tracking result. This can be understood by investigating the numerical method of SSD minimization. At a certain point in the iterative minimization, one arrives at solving the so-called *normal equations* of the form
\[
\Delta [d_k d_t] = (M^T M)^{-1} M^T \hat{e} 
\]
which results in an improvement \( \Delta [d_k d_t] \) of the parameters toward the final solution \( [d, d_t] \). The vector \( \hat{e} \) contains intensity differences between corresponding samples in frame \( t \) and \( t+dt \). Its exact structure is irrelevant for the following considerations. According to Danuser and Mazza (1996) the matrix \( M \) consists of \( n \) lines, where \( n \) is the number of intensity samples within the ROI. Every sample \( I_k(x, t) \), \( \forall k=1...n \) produces one line \( M_k \) in \( M \) which has the form
\[
M_k = \nabla I_k(x, t) \begin{bmatrix} 1 & 0 & x_k & 0 \\ 0 & 1 & -1 & x_k \end{bmatrix}.
\]
\( \nabla I_k(x, t) \) denotes the intensity gradient at the sample \( I_k(x, t) \). To produce \( M_k \) the gradient is multiplied by a matrix containing the coordinates \( x=[x_1, x_2] \) of the sample location. Importantly, if there is no local contrast \( \nabla I_k(x, t)=0 \) and consequently \( M_k=0 \). In other words, samples within the ROI which show only weak or even no contrast do not contribute to the normal equations and, therefore, have no impact on the tracking result. Obviously, the only area in the ROIs of Fig. 2 where strong contrast is observed falls along the leading edge of the tube. To summarize, because of the numerical properties of the tracker, only the image of the leading edge of the tube has influence on the path estimate. All other samples within the rectangular ROI are inherently masked by the SSD minimization.

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