The pollen tube journey in the pistil and imaging the in vivo process by two-photon microscopy. Alice Y Cheung, Leonor Boavida, Min Aggarwal, Hen-Ming Wu, and Jose A Feijo

SUPPLEMENTARY DATA

Supplemental Figure S1. The montage represents a complete sequence of optical sections (2µm) of a z-stack from the epidermal surface (ep) to a median section inside the ovary (84µm). Structural details can be revealed in the different optical sections below the epidermis (ep) the hollow ovary bears the ovules. A pollen tube can be observed crawling in the funiculus (fu), and a bright GFP signal in one of the synergids (sy) of an embryo sac (ES) indicates a neighbor fertilized ovule.
Supplemental Figure S2. The montage represents a complete sequence of optical sections (2 µm) of a z-stack from the epidermal surface (ep) to a median plane inside the ovary (32µm). A good detail of all structures is obtained, from the epidermal cells to the funiculus (fu), ovules and the fertilized embryo sacs (ES) showing a clear accumulation of GFP that spread through one of the synergid cells (sy).
Supplementary Information

Two-photon imaging of a living pistil and technical considerations

The combined use of two-photon excited (TPE) microscopy (Feijo and Moreno 2004) and a GFP expressing *Arabidopsis* line directed by a pollen-specific promoter (Twell et al., 1989) (LAT52::eGFP) was used to monitor cellular events occurring during pollen-pistil interactions. All observations were done in living tissues on intact plants without any dissection or wounding to expose tissues or organs. The flowers were emasculated 24 hours prior to pollination and left in the greenhouse until imaging. The pistil was mounted on a glass slide containing a small strip of double-sided tape to help maintain sample stability during image acquisition. Silicone grease was used as a spacer between the slide and the coverslip to avoid tissue damage or squashing. For *in vivo* pollen tube germination, the chamber was kept in a moist atmosphere using small strips of wet filter paper around the microchamber walls and sealed with fingernail polish. Imaging was performed using the internal PMT detectors and a laser power intensity of 2-5%. For imaging of pollen tube growth inside the ovary, the pistil was immersed on pollen germination medium (1% H$_3$BO$_3$, 10% Sucrose, 50 mM MES pH6, 1 mM KCl, 10 mM CaCl$_2$) in order to decrease the refractive index and increase image resolution. A deep penetration of the tissue was obtained using high power laser intensity (30-65%) and the external PMT detectors, with no observed cell damage. After imaging, plants were returned to the greenhouse for recovery and further growth. Fertilization and seed sets appeared normal.

All imaging was performed in a two-photon microscope (Bio-Rad 1024 MP), coupled to a Nikon Eclipse TE-300 inverted epifluorescent microscope, water immersion
Nikon Plan Fluor objectives with working distances on the range of 2mm (NA=1.0). For excitation we used a Coherent Mira-Verdi 900 Ti-Shapire laser tuned at 870 nm (optimized excitation for this specific GFP). The emitted fluorescence was collected after a dichroic beam splitter DCLP 550 nm. Lasersharp software was used to collect time-lapse and Z-stacks using the external non-descanned detectors 3D projections were obtained by collecting series of approximately 30 optical sections, each section with approximately 2-5 μm thick, in the Z axis, zoom 1-4x, 2-65% laser power, and using Kalman averaging of three full scans. Time-lapse images of 20 sec interval were acquired selecting 5-10 optical sections of 1-2 μm thick on Z axis using the same conditions. Image processing was performed using the software package Metamorph v6.1 (Universal Imaging Corporation). Supplementary Movies are provided as *.avi files.

Images with high contrast and good morphological definition could be readily obtained from individual pollen grains or for an overview of the complete pollinated stigma by using a lower magnification without losing much of the image resolution. Sequential optical varying from a minimum of 0.5 μm to 2-5 μm thickness could be obtained to provide 3D reconstructions of cellular or tissue structures. Moreover, the dynamics of cellular events could be followed over time with the appropriate resolution in short time intervals (e.g. 20 sec up to 1 min), depending on the number of optical sections collected. Use of higher magnifications and a lower power laser (1-2%) makes it possible to visualize cytoplasmic streaming of individual cells, e.g. pollen grains, where details of the assembly kinetics of small vacuoles inside the grain and during emergence of the pollen tube could be followed.
The limits of image resolution are determined mainly by the intensity of the GFP signal and the power laser intensity. Combined with z-stacking, these constraints sometimes led to saturated images, which could nonetheless be captured without damaging the stigmatic cells. Some difficulties may arise in attempts to monitor the dynamics of pollen tube growth inside the carpel. Pistil architecture and morphology differ slightly between Arabidopsis ecotypes and the carpels on Columbia and C24 ecotypes allowed the best imaging results, whereas in Landsberg imaging the same events was more difficult because of its considerably thicker pistil. Moreover, the highly compact arrangement of ovules along the locules and the way pollen tubes grow winding along the tissues made it hard to visualize the pollen tubes in their entirety, since the intensity of the GFP signal was masked by the ovary tissues. Furthermore, because pollen tube signal was not uniform in the entire ovary, it is left to chance that a pollen tube tip is found on the right position to be monitored over time through a long growth path. Together these add to a methodology with a relatively long learning process for the investigator and requires significant amount of time investment for preparation of samples that will yield maximum information.

The application of TPE microscopy can be extended to a variety of other optical reporters and organ or tissue types. For instance, GFP variants fused sperm or egg cell specific promoters or proteins may permit visualization of the fertilization process as it happens (e.g. Xu et al., 2002; Engel et al., 2003) or for other tissue and organ types (see for an example, Nakajima et al., 2001). Combined with emerging tools such as nanosensors developed to report cellular parameters such as pH, oxygen levels, and fluorescent reporter proteins for cytoskeleton dynamics, two-photon excitation microscopy provides
new tools to monitor how cells regulate their activities especially in the context of whole organ, or organism.

**References**


