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Visualization of meiotic events in intact living anthers by means of two-photon microscopy

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Abstract

In this paper we describe the application of two-photon microscopy (2PM) to the study of meiosis in plants. Fresh, unfixed anthers of *Agapanthus umbelatus* were briefly incubated on a minimal medium containing the DNA fluorophore DAPI. DAPI incorporation took place in about 30 min and nuclei and other DNA-containing organelles kept their fluorescence for more than 24 h. Using 2PM it was possible to optically section the whole, unfixed anthers to a depth of approximately 200 μm . This was up to the mid sagittal section and into the sporogenic tissue. Several meiotic figures were observed with unparallelled resolution. Sequences of nuclear dynamics and division were occasionally observed in the surrounding tissues and epidermal layer of cells. However we could not optimize the procedures up to the level of observing the dynamics of division on the meiotic nuclei as well. We hypothesize that either (1) meiotic cells are sensitive to the reasonably high excitation levels of infrared light needed to attain such penetration in the tissue, or (2) that our incubation procedures are not sufficiently non-invasive for meiosis to remain unperturbed. To the best of our knowledge this is the first report on direct observation of living meiotic cells in plants and establishes the potential of 2PM for intact organ research. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Meiosis; Anthers; Two-photon microscopy; DAPI; *Agapanthus umbelatus*

1. Introduction

The recent development of two-photon microscopy (2PM) has raised expectations for the observation of living phenomena in entire organs and tissues (Piston et al., 1993; Potter, 1996; Gerritsen and DeGrauw, 1999). An important candidate for this improved technology is the observation of meiotic processes. In most multicellular organisms, meiotic processes are dependent on complex interactions with surrounding tissues, and are disrupted, or largely affected, upon any kind of invasive perturbation. Some success has been obtained with Green Fluorescent Protein (GFP) incorporation into microtubules and associated proteins, which allowed the direct live observation of some prophase I events in the fission yeast (Chikashige et al., 1994; Ding et al., 1998) or late meiotic events on isolated, in vitro activated *Drosophila* oocytes (Endow and Komma, 1997, 1998). Image deconvolution (Dawe et al., 1994; Yu et al.,

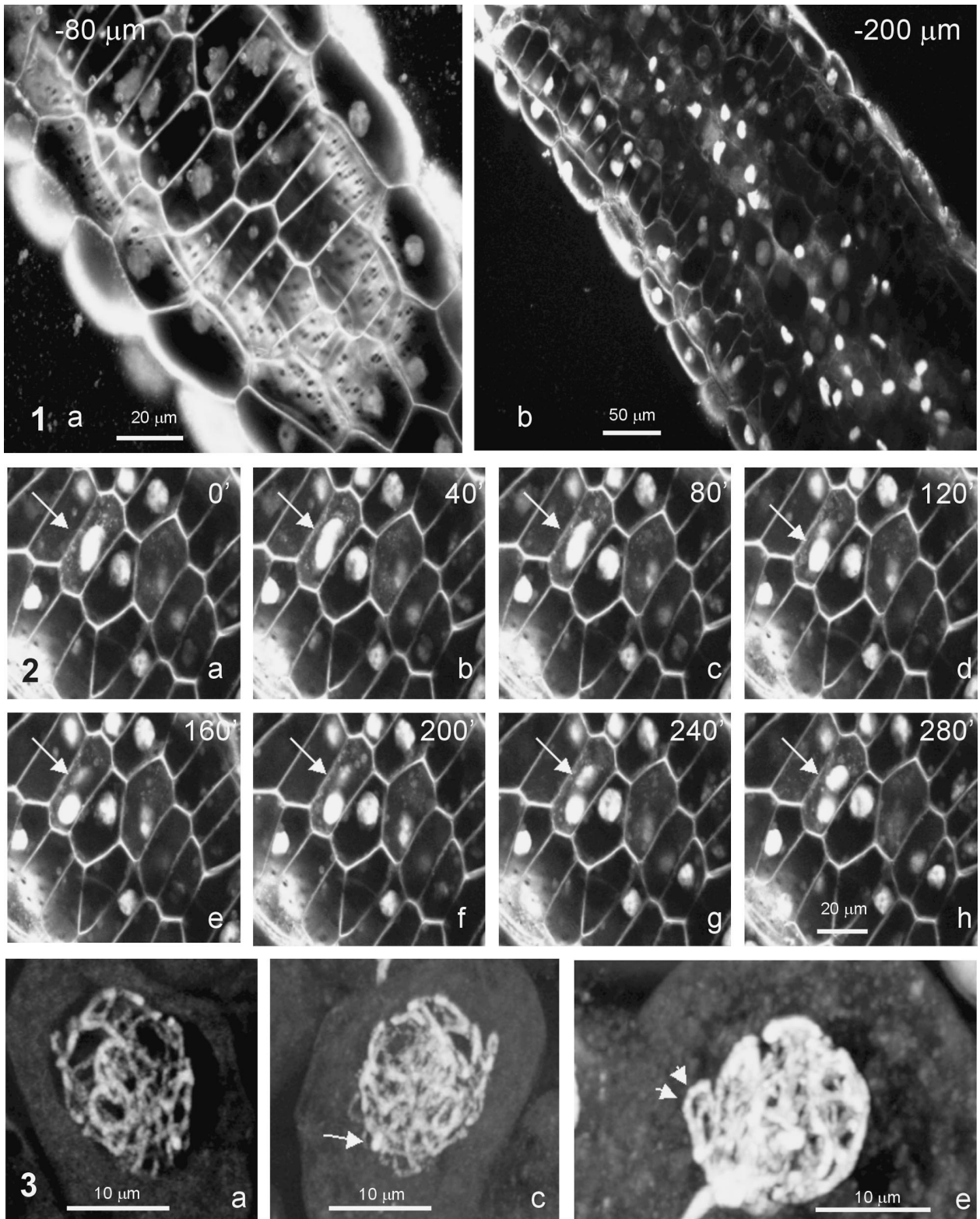
1999) and conventional confocal microscopy (Martinez-Perez et al., 1999; Franklin et al., 1999) have allowed very precise approaches to meiotic nuclei and chromosome structure, but the intrinsic dynamics of the whole meiotic process remain largely unknown. Other than these studies, most of the data on meiosis is still based on squashed or grossly sectioned material, where contradictory data has long shown possible important artifacts of these procedures (Bennet et al., 1979; Aragon-Alcaide et al., 1998). One sensible way to approach the issue would then be to directly image the meiotic cells inside the whole intact organ, under conditions as close as possible to normal physiology. Two photon microscopy, in theory, offers much better penetration while being less invasive (Piston et al., 1993; Denk et al., 1995; Cox and Sheppard, 1999). It could, therefore, provide a possible approach to studying meiosis in much less perturbing conditions.

In this paper we describe the first study of meiosis in unfixed plant anthers using 2PM. Our results clearly show that this technology has the potential to penetrate organs to a depth of 200 μm and to image nuclear and chromosome fine structure with unprecedented resolution. Although so far we could not optimize the incubation conditions up to the level

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Abbreviations: 2PM, two-photon microscopy; DAPI, 4,6-Diamidino-2-phenylindole; APW, artificial pond water



of allowing normal meiotic behavior, it is now a tangible possibility that improvements in methodology will eventually allow the entire meiotic process to be followed inside living anthers.

2. Material and methods

2.1. Anthers

Fresh anthers were collected from inflorescences of *Agapanthus umbelatus* in the correct development stage for meiosis to be occurring. Anthers were incubated on artificial pond water (APW; Miller and Gow, 1989) containing $50 \mu\text{g ml}^{-1}$ of DAPI (4,6-diamidino-2-phenylindole), and less than 0.1% DMSO (di-methyl sulphoxide) for 15–120 min. Thirty minutes incubation was found to be sufficient for DAPI labeling throughout the anther and was used thereafter. APW medium was used because it caused no gross visible changes in the volume and appearance of the anthers up until 72 h, whereas the more concentrated media used for anther culture introduce developmental changes almost immediately. Anthers were then washed twice in APW, and imaged inside culture petri dishes (WillCo-Glass Bottom Dish, GWSt-1000, Leica Comercial, Portugal) with APW. Selected anthers were observed up until 72 h, but the results presented here refer to the first 24 h, as from that point on some limited symptoms of necrosis began to be apparent in the anther filament and epidermal outer layers.

2.2. Two-photon microscopy

Imaging was carried out on a Bio-Rad 1024MP (Bio-Rad Microsciences, UK), on a Nikon TE300 Eclipse microscope (Nikon Portugal, Portugal). Objectives used were of the infinity corrected range from Nikon. Most images were collected using the PlanApo 60x, NA 1.4, oil immersion, WD 0.21 mm and PlanFluor 40x, NA 1.3, oil immersion, WD 0.2mm. Immersion oil was from Cargille (fluorescence immersion oil, Cargille Labs., Cedar Grove, NJ, USA). Two photon excitation was achieved by using a Coherent Mira-F Titanium-sapphire laser, pumped by a 5 W Verdi green laser, and using extended band X-wave optics (Coherent Laser Co., San Jose, CA, USA). For DAPI excitation, the

laser was scanned from 710 to 770 nm range, with a peak performance at ca. 740 nm. Using the bandwidth semi-quantitative method, pulse length was estimated at ca. 130 fs. First dichroic set was TSI (LP 700 nm), and all images were captured using the internal photomultipliers and emission filter 455DF35. Laser power, as measured directly from the objective with a Coherent Fieldmaster equipped with an LM-2, high sensitivity detector, ranged from 80 to 950 μW , respectively needed to go from the surface to the mid section of the anther.

2.3. Image processing

All capture, Z-section and time-sequences were controlled through Lasersharp (Bio-Rad Microsciences, UK). Three-dimensional reconstructions are volume rendered weighted projections with a lighting model applied, generated with VoxelView, (Vital Images Inc, Fairfield, Iowa, USA).

3. Results

3.1. Two-photon microscopy can image the entire fresh anther

We first addressed the question of whether 2PM could attain the kind of penetration needed to image whole anthers, an organ which in this species is between 0.2 and 1.0 mm in diameter, depending on the developmental stage. Conventional confocal microscopy cannot usually image more than 2/3 cell layers, while in the onset of meiosis, sporogenic tissue will be at least in cell layer 4, or even deeper, depending on the species and developmental stage. Fig. 1 shows that 2PM imaging of DAPI-incubated cells penetrates deep enough to allow this approach. The two images show optical section at the 2/3 cell layer interface ((a) 80 μm depth), and at the sporogenic tissue level ((b) 200 μm depth). In anthers undergoing meiosis, mid sagittal section is usually between 150 and 250 μm depth. We could resolve the meiotic cells satisfactorily at this depth, the major limitation being the working distance of the objectives. DAPI penetration and labelling was uniform throughout this volume, whereas conventional confocal nuclear dyes (propidium iodide, ethidium bromide) will

Figs. 1–3. Fig. 1. Optical sections from a Z-stack, of a whole, unfixed, *Agapanthus umbelatus* anther briefly incubated in DAPI, focussed at 80 and 200 μm from the surface, respectively. (a) Shows a tangential optical section cutting into the endothelial cells. The thickenings of the oblique cell walls are visible through their autofluorescence. Also evident are the plastids, apparently though DAPI staining of plastid DNA. At this level nuclear migration and vibrational movements (not simple Brownian motion) were frequently observed. It also shows a mid-transverse optical section through the anther. The sporogenic tissue is identified since meiocyte nuclei (in the centre region) show brighter fluorescence. Fig. 2. Time lapse sequence showing a cell division in the tapetum. Images were collected at 40 min. intervals, the sequence depicted lasting about 4–5 h. Time/space resolution was not good enough to discriminate all the steps of the mitotic process, but nuclear division is clear in the last steps (Fig. 4(g) and (h), arrows). Some of the neighbouring nuclei also display small movements along the sequence. This cell was followed 4 h afterwards, but no evidence of cytokinesis was observed. Fig. 3. Zoom of the sporogenic tissue, illustrating early, mid and late-prophase nuclei. Consecutive thickening of chromosomes is clearly visible as nuclei maintain their regular structure/size. In mid and late prophase (Fig. 2(b) and (c)) chiasmata are sometimes distinguishable (arrows).

not cross the callose layer between the tapetum and the meiocytes (Feijó, 1995).

In Fig. 1(a) the autofluorescence of the cell walls of the endothecium reveals the architecture of plasmodesmata and wall thickening deposition, seen here in a tangential optical section. Also clearly visible are the plastids, presumably by DAPI staining of their DNA, since they have no pigments in this tissue and are not visible in unstained preparations. Fig. 1(b) shows the deepest optical section attainable with the optics used (PlanApo 60x, NA1.4, oil, WD 0.21 mm). The brightly stained nuclei in the core of the section are the meiotic cells, here in early prophase I. While providing the best working distance (WD), the specific objective used for this section required a significant level of excitation (close to 1 mW). It should be stressed that, even with PlanApo lenses, power was never stretched to detectable fading levels, nor was it necessary to use the more sensitive, but less convenient, external non-descanned detectors (Piston and Knobel, 1999).

3.2. Cell viability is maintained under two-photon excitation

The second point we wished to verify was the non-invasive nature of the imaging process. The effort of imaging the whole organs is only worthwhile if the natural cell dynamics can be followed. The results obtained up to this point should still be considered as preliminary, but some temporal sequences, such as the one shown in Fig. 2, clearly indicate that this is achievable. In this sequence, images were captured at 40 min intervals from 0 to 16 h. The tapetum cell depicted (arrow) underwent a process of nuclear division that lasted about 4 h. The resolution of the mitotic steps is not clearly visible, but the binuclear stage of the cell is in Fig. 2(h). This sequence continued for more than 3 h but we could not detect evidence of cytokinesis. However, the tapetum frequently develops into coenocytic multi-nucleate structures, so lack of cytokinesis may not necessarily imply perturbation by the imaging process. Other signs of cell vitality included widespread observation of nuclear movement, rotations and streaming of organelles, during periods up to 16 h. Unfortunately this was not the case inside the sporogenic tissue, and no evidence of nuclear dynamics could be clearly observed in the meiotic nuclei.

3.3. High resolution of meiotic nuclei and chromosomes inside the whole anther

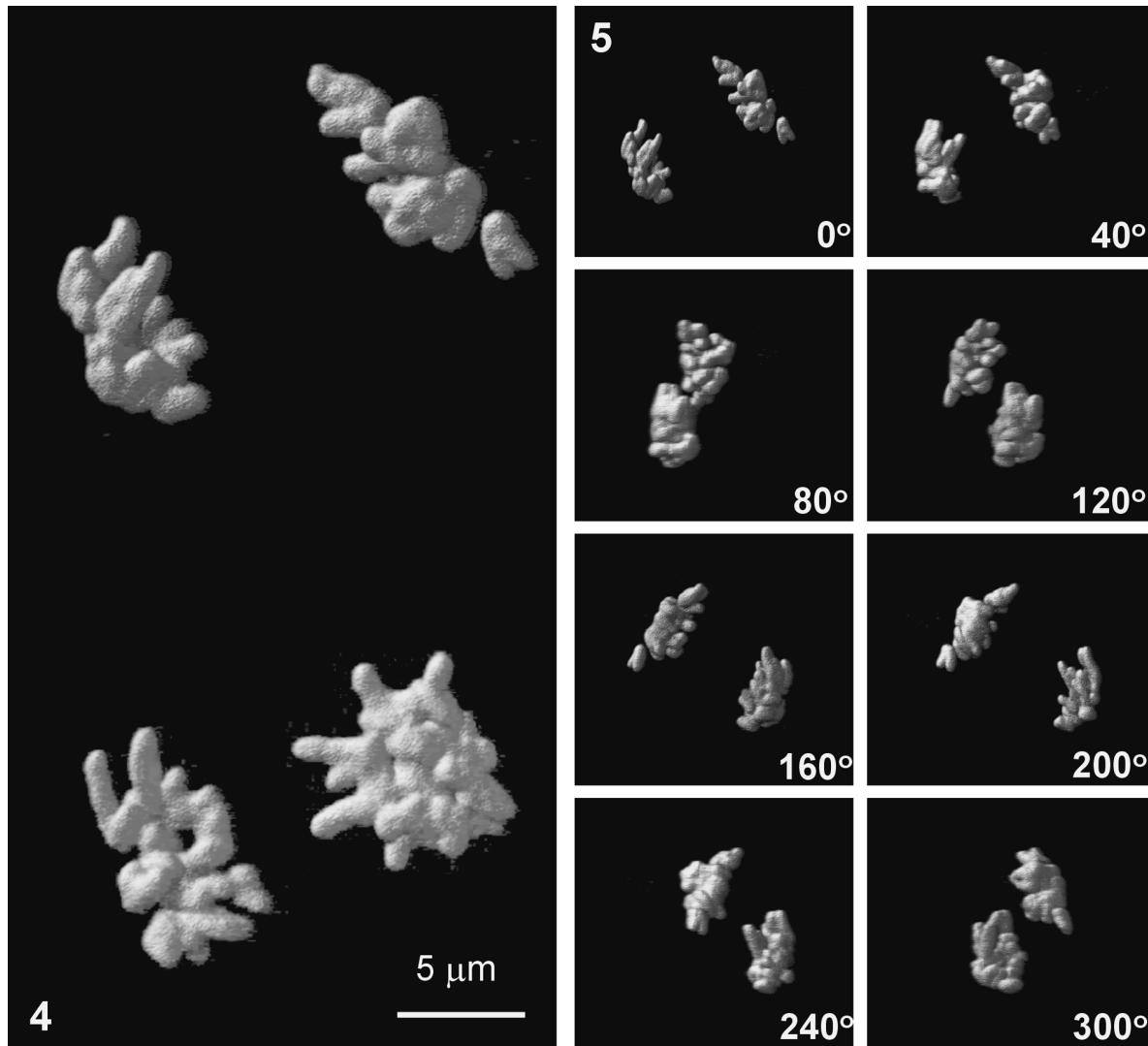
While the incubation and imaging techniques were not optimized up to the level of imaging nuclear dynamics of meiocytes, we addressed the capacity of 2PM to produce high-resolution images at such depth. Figs. 3 and 4 clearly show that this is the case. Fig. 3 depicts the progression of prophase I ((a)–(c) early mid and late), with several chiasmata clearly resolved (arrows). Fig. 4 shows a volume rendering of a three dimensional dataset of a cell undergoing anaphase II. Chromosome structure and external texture are clearly resolved. A 3-D full rotation of the upper spindle is

shown in Fig. 5. Chromosome arrangement, thickness and relative positioning of the chromosomes are all clearly resolved.

4. Discussion

In this paper, we firstly aimed to determine the applicability and possible advantages of two-photon microscopy (2PM) for the study of meiosis in a situation as close as possible to the true biological context. Our results show that this point is sufficiently well substantiated from the optical and technical point of view, since 2PM could easily produce high resolution images down into the sporogenic tissue. In doing so, to the best of our knowledge, we describe the first approach to meiosis on intact, unfixed, living, plant anthers. Our results clearly show that this technology has the potential to penetrate into organs of this range of size, and to image nuclear and chromosome fine structure with unprecedented resolution. The level of penetration we attained was limited not by illumination power, nor heat-induced structural damage, but by the objective working distance, suggesting that deeper penetration is attainable if longer working distance objectives of comparable numerical aperture become available. This constitutes a major improvement in the currently available techniques for studying structural re-organization meiotic nuclei, an issue of paramount biological relevance. To date most of the relevant data is still generated by squash/extrusion approaches (e.g. Bai et al., 1999; Yu et al., 1999; Franklin et al., 1999; Couteau et al., 1999). This type of preparation has been shown to induce various kinds of structural artifacts when compared with material prepared by fixation and sectioning or confocal imaging (Bennet et al., 1979; Aragon-Alcaide et al., 1998). This has important consequences for conclusions about such matters as pairing and the relative positions of chiasmata.

In plants, the existence of a large degree of cytoplasmic connection between male meiocytes by the so-called “cytomictic channels” (Heslop-Harrison, 1966), through which organelles and even nuclear material (“cytomixis”) may be exchanged between different meiocytes (Feijó and Pais, 1989), renders any kind of mechanical and even chemical manipulation as a putative artifact inducer (Feijó, 1995). In this paper we demonstrate that 2PM may be a possible circumvention of these invasive procedures. The degree of detail and the “physiological” appearance of prophase nuclei and the perfect preservation of size, position and external morphology of chromosomes after volume rendering (Figs. 4 and 5) are unprecedented for unfixed samples. Similar levels of resolution and three-dimensional analysis have been attained with image deconvolution systems (Dawe et al., 1994) but only on material prepared by fixation and meiocyte extrusion. While many experiments still rely on fluorescent in-situ hybridization (FISH) and immunolocalization, thus by necessity implying some degree of



Figs. 4 and 5. Fig. 4. Volume rendering of a metaphase II double spindle. Chromosome organization and relative position are clearly visible. Fig. 5. Full loop rotation of the upper spindle. Lateral projection shows that the architecture of each chromosome set is not exactly symmetrical (evident after 120° rotation). Rotation also shows an “orphan” chromosome in one of the sets (especially evident in the 160° rotation).

invasiveness, the advent of GFP and related fluorescent proteins makes it possible that many of such studies may in the future rely on the utilization of 2PM.

From the biological point of view we could not, however, optimize visualization to the point of observing vital signs in the sporogenic tissue, specifically meiotic progression of the cells. Nuclear vibration and movement (clearly not just Brownian motion) were consistently observed in the neighboring tissues and occasionally cell division (but never cytokinesis). However in the sporogenic tissue these kinds of movements and progression were never observed. At this point we can only speculate about two possible causes for this failure. On one hand the infrared power needed to penetrate such thick specimens most of the time falls close to the mW range. Most of our observations were done in the 720–740 nm range (optimal for DAPI) at which possible absorption by specialized plant cell receptors/molecules can not be

excluded. The amount of energy irradiated is also not negligible. Higher-order photon interactions in 2PM imaging have recently been revealed by photobleaching in thin samples (Patterson and Piston, 2000) showing that complex interactions and generation of radicals may be occurring. This could have important biological consequences, partially contradicting early optimistic views about the non-invasiveness of 2PM (Potter, 1996). This is certainly the case also for wild-type GFP, for which one-photon excitation seems to provide higher stability and reproducibility, even in thick organs such as *Arabidopsis* roots (unpublished data). The second possible explanation relates to the simple incubation protocol we used. Certainly APW did not induced major alterations to the size or morphology of the anthers, but observations after 3–4 days showed that many cells were becoming necrotic. This fact points out the necessity of optimizing a better culture medium, an

objective within reach since anthers have been cultured from meiosis up until viable pollen formation (Reynolds, 1997; Heberle-Bors, 1998), albeit not in conditions permitting microscope observation. Isolation of anthers from the flower might also induce injury responses that block meiosis.

In conclusion, while from the biological point some optimization of methods is still required, technically we prove that 2PM offers the potential to observe, for the first time, meiotic progression in multi-cellular organisms on a non-invasive, non-disruptive form.

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References

- Aragon-Alcaide, L., Beven, A., Moore, G., Shaw, P., 1998. The use of vibratome sections of cereal spikelets to study anther development and meiosis. *Plant J.* 14, 503–508.
- Bai, X., Peirson, B.N., Dong, F., Xue, C., Makaroff, C.A., 1999. Isolation and characterization of SYNI, aRAD21-like gene essential for meiosis in *Arabidopsis*. *Plant Cell* 11, 417–430.
- Bennet, M.D., Smith, J.B., Simpson, S., Wells, B., 1979. Intranuclear fibrillar material in cereals pollen mother cells. *Chromosoma* 71, 289–332.
- Chikashige, Y., Ding, D.-Q., Funabiki, H., Haraguchi, H., Haraguchi, T., Mashiko, S., Yanagida, M., Hiraoka, Y., 1994. Telomere-led premeiotic chromosome movement in fission yeast. *Science* 264, 270–273.
- Couteau, F., Belzile, F., Horlow, C., Grandjean, Vezon D., Doutriaux, M.-P., 1999. Random chromosome segregation without meiotic arrest in both male and female meiocytes of a *dmcl* mutant of *Arabidopsis*. *Plant Cell* 11, 1623–1634.
- Cox, G.C., Sheppard, C.J.R., 1999. Multiphoton fluorescence microscopy. In: Mason, W.T. (Ed.). *Fluorescent and Luminescent Probes for Biological Activity*. 2nd ed. Academic Press, pp. 331–336.
- Dawe, R.K., Sedat, J.W., Agard, D.A., Cande, W.Z., 1994. Meiotic chromosome pairing in maize is associated with a novel chromatin organization. *Cell* 76, 901–912.
- Denk, W., Piston, D.W., Webb, W.W., 1995. Two photon molecular excitation in laser scanning microscopy. In: Pawley, J.B. (Ed.). *Handbook of Biological Confocal Microscopy*. 2nd ed. Plenum Press, New York, pp. 445–458.
- Ding, D.-Q., Chikashige, Y., Haraguchi, T., Hiraoka, Y., 1998. Oscillatory nuclear movement in fission yeast meiotic prophase is driven by astral microtubules, as revealed by continuous observation of chromosomes and microtubules in living cells. *J. Cell Sci.* 111, 701–712.
- Endow, S.A., Komma, D.J., 1997. Spindle dynamics during meiosis in *Drosophila* oocytes. *J. Cell Biol.* 137, 1321–1336.
- Endow, S.A., Komma, D.J., 1998. Assembly and dynamics of an anastral:astral spindle: the meiosis II spindle of *Drosophila* oocytes. *J. Cell Sci.* 111, 2487–2495.
- Feijó, J.A., Pais, M.S., 1989. Cytomixis in meiosis during the microsporogenesis of *Ophrys lutea*: an ultrastructural study. *Caryologia* 42, 37–48.
- Feijó, J.A., 1995. Sexual reproduction in *Ophrys lutea*. PhD. Thesis, University of Lisboa, Lisboa.
- Franklin, A.E., McElver, J., Sunjevaric, I., Rothstein, R., Bowen, B., Cande, W.Z., 1999. Three-dimensional microscopy of the rad51 recombination protein during meiotic prophase. *Plant Cell* 11, 808–824.
- Gerritsen, H.G., DeGrauw, C.J., 1999. Imaging of optically thick specimen using two-photon excitation microscopy. *Microsc. Res. Tech.* 47, 206–209.
- Heberle-Bors, E., 1988. Experimental control of pollen development. In: Chupeau, Y., Caboche, M., Henry, Y. (Eds.). *Androgenesis and Haploid Plants*. Springer-Verlag, Berlin, Heidelberg, New York.
- Heslop-Harrison, J., 1966. Cytoplasmic connections between angiosperm meiocytes. *Ann. Bot.* 30, 222–230.
- Martinez-Perez, E., Shaw, P., Reader, S., Aragon-Alcaide, L., Miller, T., Moore, G., 1999. Homologous chromosome pairing in wheat. *J. Cell Sci.* 112, 1761–1769.
- Miller, A.L., Gow, A.R., 1989. Correlation between profile of ion-current circulation and root development. *Physiologia Plantarum* 75, 102–108.
- Patterson, G.H., Piston, D.W., 2000. Photobleaching in two-photon excitation microscopy. *Biophys. J.* 78, 2159–2162.
- Piston, D.W., Knobel, S., 1999. Quantitative imaging of metabolism by two-photon excitation microscopy. *Methods in Enzymology* 307, 351–369.
- Piston, D.W., Summers, R.G., Webb, W.W., 1993. *Biophysical Journal* 63, A110.
- Potter, S., 1996. Vital imaging: two photons are better than one. *Curr. Biol.* 6, 1595–1598.
- Reynolds, T.L., 1997. Pollen embryogenesis. *Plant Molecular Biology* 33, 1–10.
- Yu, H.-G., Muszynski, M.G., Dawe, R.K., 1999. The maize homolog of the cell cycle checkpoint protein MAD2 reveals kinetochore substructure and contrasting mitotic and meiotic localization patterns. *J. Cell Biol.* 145, 425–435.