

TECHNICAL ADVANCE

# The use of vibratome sections of cereal spikelets to study anther development and meiosis

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## Summary

**We have developed a method using vibratome sectioning coupled with confocal microscopy to analyse pre-meiotic and meiotic events in developing wheat anthers. This report describes the application of fluorescence *in situ* hybridization to three-dimensionally preserved tissue sections from intact florets. We show that excellent imaging of all the stages of meiosis up to telophase I can be achieved, and show centromere positions and pre-meiotic homologous pairing both in meiocytes and tapetum cells. These clear results using intact tissue sections are contrasted with conventional spreading methods for analysis of meiosis in plants, which we show can produce artefactual or uninterpretable results.**

## Introduction

Most labelling techniques in cell biology represent a compromise between optimal preservation of cell and tissue structures and accessibility of those structures and their constituent macromolecules to labelling reagents. Many animal cell biological studies use cultured fibroblasts and other cultured cells which are naturally flat and spread in monolayers. In contrast, most plant cells have considerable depth, often being close to isodiametric, and studying them in detail at the optical level requires confocal or other 3-D microscopical techniques. Furthermore, many of the most interesting aspects of plant cell biology and plant development occur in cells deep within the plant. For most plant species the interior cells below the subepidermal layer are difficult or impossible to image even by confocal microscopy. Thus, some form of dissection or sectioning is necessary.

Meiosis in angiosperms takes place within the complex structure of developing flowers (for reviews see Esau, 1977; Goldberg *et al.*, 1993). Male meiosis occurs during anther development and has been studied most often, since the immature anthers are quite easily dissected out, whereas

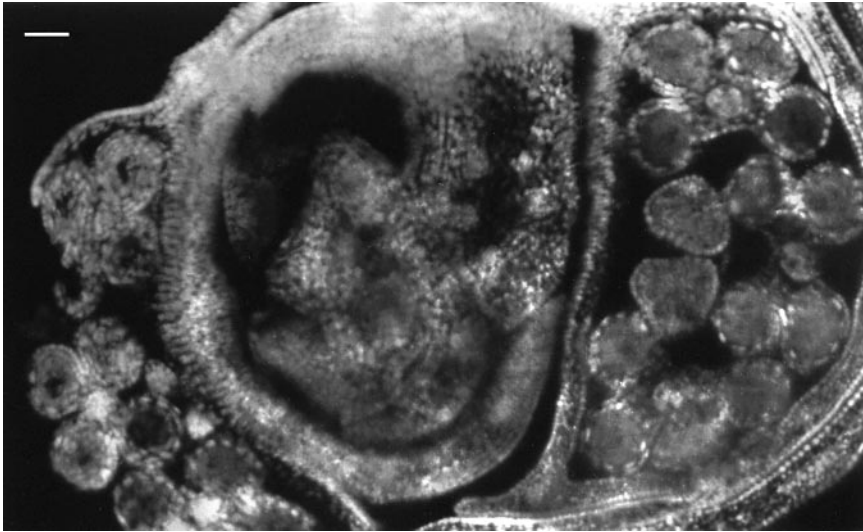
the developing female ovules are considerably less accessible. Thus, cytogeneticists have typically studied meiosis by dissecting anthers, and then cutting or squashing them to release the meiocytes. This causes at least two problems. First, the cellular and nuclear structure is considerably disrupted. Since this is necessary for many cytological studies of metaphase chromosomes, which require chromosome squashing and spreading, it is often not thought to be a serious problem. However, the three-dimensional structure of the nuclei will certainly be altered and so no reliable conclusions can be drawn about nuclear or chromosomal organization in such preparations. Second, the tissue morphology and thus the relation of the various cells to one another is lost. This makes it impossible to categorize the different cell types that are dispersed from the anther. Again, this is not a problem when studying identifiable stages of meiosis, since once the characteristic chromosome condensations of meiotic prophase are visible, the identity of the cell or nucleus as a meiocyte is obvious. For stages of development preceding meiosis, where characteristic chromosomal structures are not present, this is a serious problem.

We have been studying early stages development of male meiocytes and their precursors in hexaploid wheat, and have found the standard techniques at best unhelpful and at worst misleading. We describe here a straightforward method we have developed to study the early stages of anther and meiocyte development by a combination of thick sectioning using a vibratome and confocal microscopy. We have used various types of *in situ* labelling, immunofluorescence labelling and BrUTP transcription labelling on these sections. We have demonstrated that all of the stages of meiosis I (prophase–telophase) can be followed in this type of section.

## Results and discussion

Initially, we attempted to cut vibratome sections of dissected anthers. While it was possible to obtain longitudinal sections with difficulty, it proved impossible to cut transverse sections because the anthers were not rigid enough to section cleanly, and because the resulting sections did not remain intact. However, transverse sections are essential to see the morphology of the anther and locules. Sectioning of the whole spikelet was much easier and more successful. It was initially surprising how well such sections held together, even using unfixed tissue. This is

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**Figure 1.** Low magnification confocal image of a spikelet vibratome section stained with DAPI.

Transverse sections through two florets, including several anthers, each comprising four locules can be seen. Bar = 100  $\mu$ m.

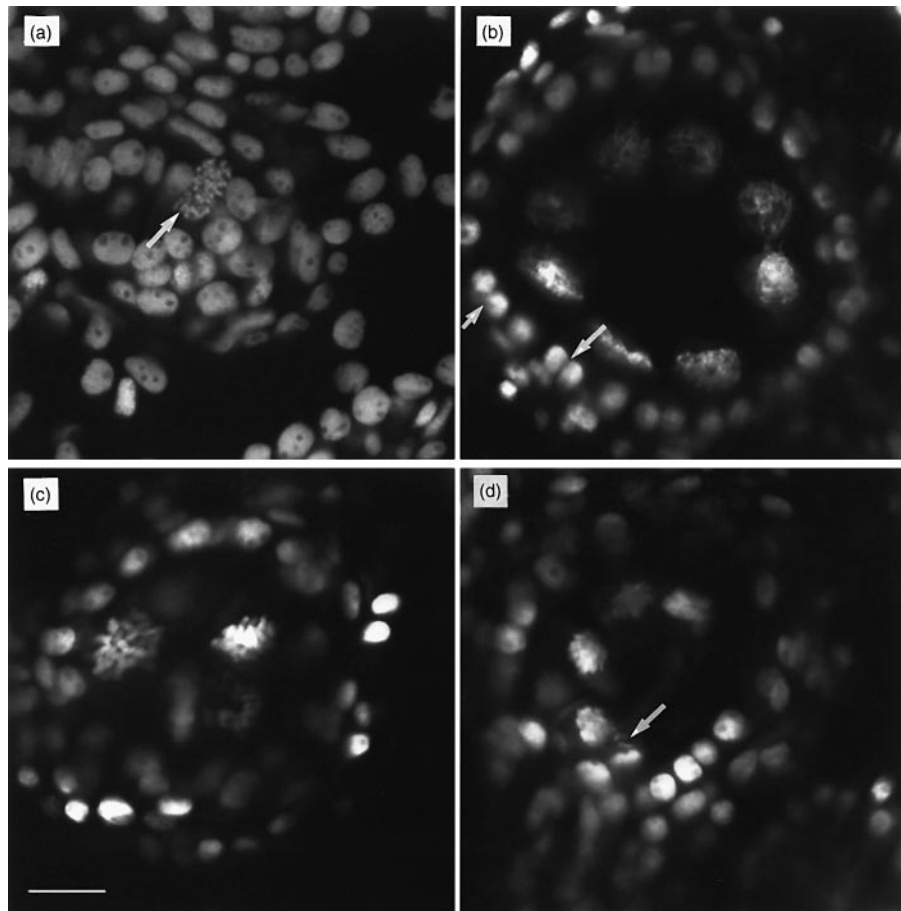
probably due in part to the fact that the young anther sacs are filled with a matrix of protein and/or polysaccharide material. In older anthers, at the tetrad and later stages, the cells from within the locules tended to be lost during sectioning, presumably because the pollen precursors become progressively more separated from each other and the surrounding cells. However, we have obtained good intact sections showing all the stages of meiosis I. Figure 1 shows a low magnification confocal image of a transverse section through a spikelet. Parts of two florets are visible, and the anthers, locules and other flower parts are easily distinguished.

Figure 2 shows a series of confocal images through anthers at different stages before and during meiosis. In each case, an entire locule comprising the central meiocytes (microsporocytes) and the surrounding tapetal cell layer is shown. In Figure 2(a) a cell in metaphase can be seen within a locule. At this stage in anther development the meiocyte and tapetum precursor cells are not distinguishable. In Figure 2(b) the meiocytes are at approximately zygotene and the surrounding tapetum cells are binucleate (arrows). In Figure 2(c) the meiocytes are at metaphase I, and in Figure 2(d) at anaphase/telophase I (a pair of lagging chromosomes is arrowed). In the latter three images the surrounding tapetum cells are clearly distinguishable from the central meiocytes.

Various types of fluorescence labelling have been successfully carried out on these thick sections. We show here the results of fluorescence *in situ* hybridization, but we have used similar methods for immunofluorescence labelling and for incorporation of BrUTP to localize transcription sites in the meiocytes and surrounding cells. Genomic *in situ* fluorescence hybridization was used on a wheat line in which one pair of wheat chromosomes is substituted with the equivalent barley

homologues. Figure 3 shows successive image planes through a confocal image stack. Figure 4(a) shows a larger region of this anther, including the whole of the relevant locule. The genomic barley label is shown in green in Figure 3(a–f). The wheat centromeres were also labelled (red/orange in a–f). In Figure 3(g–l) the corresponding image planes are shown counterstained with DAPI to show the nuclei. The full results of this study have been published elsewhere (Aragon-Alcaide *et al.*, 1997). Here, we note that at this stage of anther development, which is clearly pre-meiotic from the DAPI staining and nuclear morphology, the centromeres are clustered together in each cell at the nuclear periphery. In virtually all the cells shown, the two barley chromosomes are imaged as a single line of fluorescence labelling. This implies that the barley homologues have become co-localized at a stage well before the onset of meiosis. Furthermore, the homologues in the surrounding tapetal cells have also become co-localized.

For comparison with standard spreading methods, we show in Figure 4(b,c) a cell spread comparatively gently, from a dissected anther and labelled simultaneously with total genomic barley probe (green in Figure 4b), with probes to the centromeres (larger red spots clustered in the lower part of the nucleus in Figure 4b) and the telomeres (smaller red spots in the upper half of the nucleus in Figure 4b) using conventional methods. Although this nucleus was not intentionally squashed, it has clearly spread and flattened considerably. The centromeres and telomeres are nicely separated and can be clearly distinguished. However, the structural distortions which have plainly occurred during specimen preparation cast doubt on the interpretation of the labelling patterns. It is not possible to tell whether the two barley chromosomes, which are lying close together,

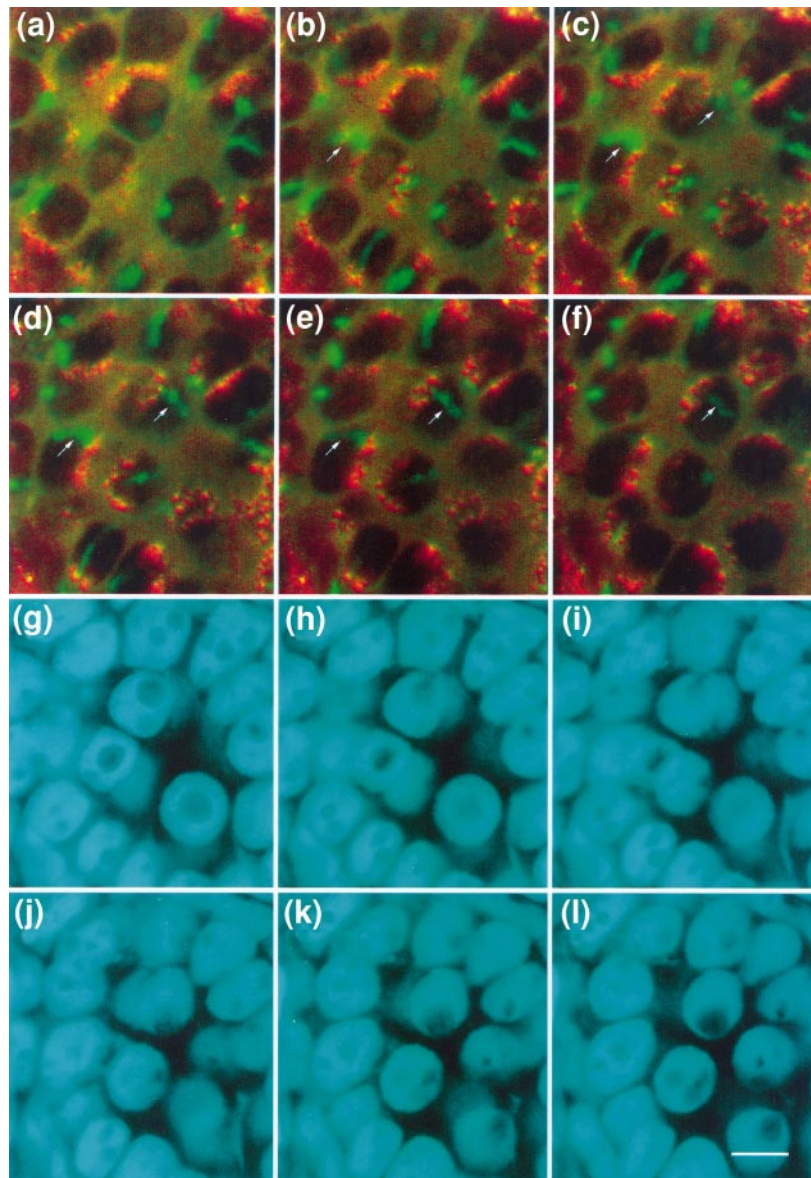


**Figure 2.** Single confocal sections from vibratome sections of spikelets at various stages before and during meiosis. In each case the nuclei and chromosomes have been stained with DAPI, and a single locule is shown. (a) An anther well before meiosis. At this stage the cells which will give rise to the microsporocytes and the precursors to the tapetum cells are not distinguishable within the structure. A metaphase cell is arrowed. (b) A ring of eight meiocytes approximately at zygotene, and the surrounding binucleate tapetal cells (arrows). (c) Meiocytes at meiotic metaphase I. (d) Meiocytes at meiotic anaphase/telophase I. A pair of lagging chromosomes can be seen (arrows). Bar = 20  $\mu\text{m}$ .

were originally associated or not before squashing. In similar preparations we have seen varying degrees of association of the labelled chromosomes at various chromosomal locations and variable degrees of chromosome condensation, none of which is reliably interpretable. In the nucleus shown, the nucleolus cannot even be distinguished (compare the clearly visible nucleoli in the intact tissue sections). Most important of all, the identity of this cell is in doubt. Before the onset of meiosis and the appearance of characteristic chromosome patterns, there is no way of determining whether the nucleus is from a meiocyte or from a neighbouring tapetal cell. Even if one could be sure that the homologous chromosomes were associated in meiocytes, the results from three-dimensionally well-preserved tissue have shown this also happens in the tapetum cells.

In summary, reliable conclusions about nuclear and cellular organization are critically dependent on adequate

cell and tissue fixation and preservation, and in most cases the maintenance of intact tissue organization is essential in the analysis and identification of developmental stages and cell types. In the analysis of early events in meiosis and anther development, traditional dissection and spreading/squashing clearly fail to meet these criteria and have the potential to produce artefactual results, to lead to misinterpretation, or to lose important structural information. We have described a straightforward experimental approach to analysing the organization of anther and floral development, which can encompass fluorescence *in situ* hybridization and immunofluorescence labelling, as well as 'semi *in vivo*' techniques such as transcription assays by BrUTP incorporation. In this communication we have presented results obtained from wheat, but we have extended this approach without difficulty to other cereal species and to at least two dicot species.



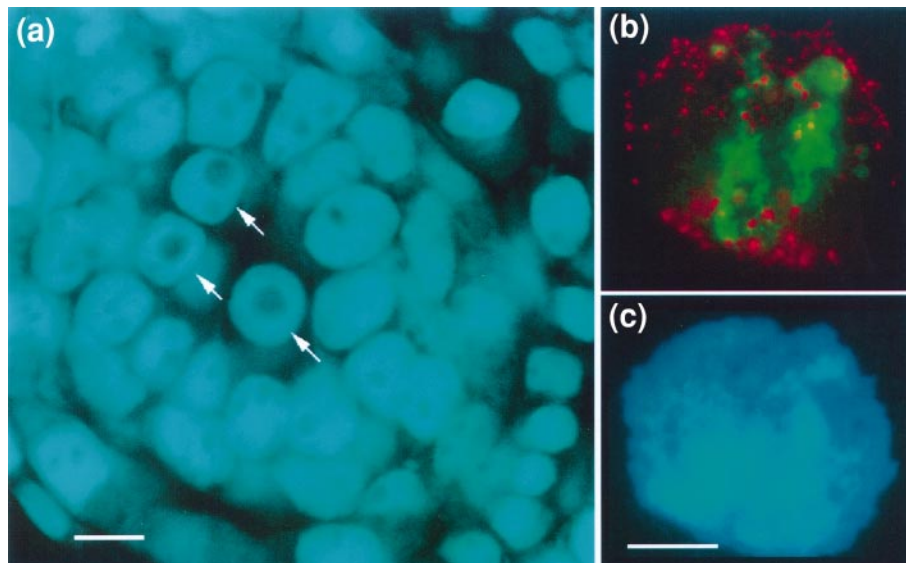
**Figure 3.** (a–f) Series of confocal sections from a vibratome section *in situ* labelled with total genomic barley probe (green) and centromere probe (orange). A region at the centre of a locule including meiocytes (see arrows in 4a) and surrounding tapetum cells is shown. In each of the cells shown, the barley homologues are visible as a single line of fluorescence, indicating that they are co-localised. The upper arrows in (c–f) trace a homologous pair through a meiocyte nucleus, and the lower arrows in (b–e) a homologous pair through a tapetum cell nucleus. (g–l) The equivalent DAPI images to (a–f). The DAPI labelling shows that the meiocytes are in pre-meiotic interphase. Spacing between consecutive confocal sections = 1.5  $\mu\text{m}$ . Bar = 10  $\mu\text{m}$ .

### Experimental procedures

Methods were developed from those described by Shaw *et al.* (1995). Developing spikes were harvested from wheat plants and the covering leaves were removed. The spikes were fixed entire in 4% (w/v) formaldehyde, freshly made from paraformaldehyde, in PEM buffer (PEM: 50 mM PIPES/KOH (pH 6.9); 5 mM EGTA; 5 mM MgSO<sub>4</sub>) to which 0.05% (w/v) Nonidet NP40 had been added. Vacuum infiltration was used to aid the penetration of the fixative solution into the air spaces. After fixing for 1 hour, the spikes were washed in several changes of PEM buffer over a period of approximately 1 hour. Individual spikelets were then dissected from the spike. The position of each spikelet along the spike was

noted. Adjacent spikelets were staged by dissecting anthers from them and squashing onto slides and staining with aceto-carmin in the conventional way (Macgregor and Varley, 1983). For vibratome sectioning, the base of the spikelet was carefully cut off perpendicular to the axis of the spikelet, and the spikelet was mounted on this cut surface using superglue onto the vibratome stage. Transverse sections approximately 100  $\mu\text{m}$  thick were then cut under water, placed on to slides coated with glutaraldehyde activated  $\gamma$ -aminopropyl triethoxy silane (APTES) and allowed to air dry. Sections of this thickness contained 2–3 layers of cells.

A vibratome sections material by means of a knife blade (we routinely use a Wilkinson Sword razor blade) which is rapidly vibrated or oscillated back and forth in the plane of the blade (i.e.



**Figure 4.** (a) Confocal image of the DAPI labelling for the entire locule from which Figure 3 was taken. Three meiocytes are arrowed. Bar = 10  $\mu$ m.

(b) Nucleus spread from a dissected anther, and *in situ* labelled by conventional methods with total genomic probe to the barley homologues (green), and to telomeres and centromeres (red). The telomeres correspond to the smaller red spots in the upper part of the nucleus, the centromeres to the larger spots clustered in the lower part. Although the barley homologues appear to be represented by two elongated regions of labelling, it is not possible to tell whether they were associated or not before disruption due to spreading and specimen preparation.

(c) DAPI staining corresponding to (b). The nucleus is considerably spread compared to equivalent nuclei in well preserved tissue sections (compare Figure 4a), and the nucleolus or nucleoli can no longer be seen. The different degrees of chromatin condensation visualised as different intensities of DAPI staining seen may represent an aspect of the original nuclear structure or may be artefactual. It is not possible to tell whether this nucleus is from a meiocyte or from a tapetal cell. Bar = 10  $\mu$ m.

the horizontal plane in the vibratomes we have used). As the blade vibrates, it is moved forward into the tissue sample, which is held on a platform. After each cut, the platform can be raised to cut the next section. The rapid vibratory movement of the blade means that many unembedded or even living tissues can be successfully sectioned down to thicknesses of approximately 50  $\mu$ m. Sectioning is carried out under water or any suitable buffer solution. We have found the Vibratome 1000 model (Technical Products International, Inc, St Louis, Missouri, USA) the most successful of those we have tested.

*In situ* hybridization and probe preparation were carried out according to protocols modified from Aragon-Alcaide *et al.* (1996). Sections were permeabilized by incubation with 2% (w/v) cellulase (Onozuka R-10) in TBS (TBS: 25 mM Tris/HCl (pH 7.4), 140 mM NaCl, 3 mM (KCl) for 1 hour at room temperature. The slides were then washed in 2  $\times$  SSC (SSC: 150 mM NaCl, 15 mM sodium citrate). Slides were treated with DNase-free RNase, dehydrated in an ethanol series (70%, 90%, 100%, 3 min each step) and air dried. The hybridization mixture, consisting of 50 ng of DNA probe and 800 ng of sheared salmon sperm DNA in 50% formamide, 10% dextran sulphate, 0.1% SDS, and 2  $\times$  SSC, was denatured on a heating block at 100°C for 5 min then rapidly chilled on ice prior to addition to each slide. The probes were applied to the tissue sections (50  $\mu$ l/slide), covered with a plastic coverslip, and then transferred to a Hybaid Omnigene machine (a modified thermocycler). The programme for chromosome denaturation was 10 min at 75°C, then 1 minute at 50°C, then 2 min at 40°C, and finally 37°C. After overnight hybridization at 37°C, slides were washed in 20% formamide in 0.1  $\times$  SSC at 42°C for 10 min, a stringency corresponding to a sequence identity of 85–90%. This was followed by washing at 42°C in 0.1  $\times$  SSC for 10 min, then 2  $\times$  SSC at 42°C

for a further 5 min, then cooling to room temperature. The sections were washed with 4  $\times$  SSC, 0.1% Tween 20, 5% BSA. This was followed by fluorescent secondary antibody probe detection of hybridization sites by either FITC-conjugated sheep antidigoxigenin antibody (Boehringer Mannheim) or Cy3-conjugated extravidin (Sigma) in 4  $\times$  SSC, 0.1% Tween 20, 5% BSA for digoxigenin or biotin labelled probes, respectively. Slides were then counterstained for 1 minute with 1  $\mu$ g ml<sup>-1</sup> DAPI (4',6-diamidino-2-phenylindole) and mounted in antifade solution (Vectashield).

Confocal optical section stacks were collected using a Biorad MRC-1024 UV confocal scanning microscope as described previously (Shaw *et al.*, 1995). Images were transferred to a Macintosh computer and assembled into composite images using Adobe Photoshop and NIH-Image, a public domain program for the Macintosh written by Wayne Rasband and available via anonymous ftp from zippy.nimh.nih.gov.

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