TITLE
Fluorescence in situ hybridization (FISH) on vibratome sections of plant tissues

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KEYWORDS
In situ hybridization, confocal microscopy, meiosis, plant chromosome association, nuclear structure, imaging
ABSTRACT
This protocol describes the application of fluorescence in situ hybridization (FISH) on three-dimensionally preserved tissue sections derived from intact plant structures such as roots or florets. The method is based on the combination of vibratome sectioning with confocal microscopy. The protocol provides an excellent tool to investigate chromosome organization in plant nuclei in all cell types and has been used on tissues of several different plant species. The visualization of three-dimensionally well-preserved tissues means that cell types can be confidently identified. For example meiocytes can be clearly identified at all stages of meiosis and can be imaged in the context of their surrounding maternal tissue. FISH can be used to localize centromeres, telomeres and sub-telomeric regions, and total genomic DNA can be used as probes to visualize introgressed chromosomes or chromosome segments.
INTRODUCTION

Many of the most interesting aspects of plant cell biology and development occur in cells deep within tissues of the plant. Examples are male meiosis occurring within the anthers and embryogenesis and endosperm development occurring within the developing seed. These cells are difficult or impossible to image, even within confocal microscopy, which is rarely able to image deeper than 100µm. Thus to be accessible to imaging, dissection or sectioning must be used. Vibratome tissue sectioning is a simple way to produce relatively thick sections (20-50µm) which can be imaged to reveal the three-dimensional structure of the underlying tissues. It has the advantage of preserving 3D structure well, so that subcellular structures can be reliably imaged. Furthermore, reliable identification of cell types often requires an accurate assessment of the tissue context, which is lost when cytological squash preparations are made.

This method does not require embedding of tissue wax or resin, and can be applied to fixed or unfixed tissues. The only requirements are that the plant tissue has to be sufficiently rigid for sectioning, and the plant parts have to be large enough to handle in the vibratome. These requirements are met by the roots and florets of many plants. We have used it for both roots and florets of wheat (Triticum aestivum L.) and related species (Aragon-Alcaide et al., 1998; Martinez-Perez et al., 2001; 2001), to rye (Secale cereale L.) florets, and to rice (Oryza sativa L.) roots and florets (Prieto et al., 2004b;c) and maize (Zea mays L.) roots (Shaw et al., 1998) among the cereals, and to roots of Pea (Pisum sativum L), bean (Vicia faba L.) (Rawlins et al., 1991), soya (Glycine max L.) (Bunney et al., 2000). The only plant species with which we have been unsuccessful in vibratome sectioning is Arabidopsis thaliana, because of its small size. By carrying out the sectioning in 100% ethanol, we have also been able to apply vibratome sectioning to early stage wheat endosperm (Wegel et al., 2005). The resulting vibratome sections can be used in many different types of labelling experiment. We have used such sections for DNA fluorescence in situ hybridization (FISH), for RNA FISH to detect transcripts, for antibody immunofluorescence labelling, and for labelling of nascent transcripts by BrUTP incorporation in unfixed tissue. We have also combined antibody and BrUTP labelling with FISH. (e.g Thompson et al., 1997).

This type of sectioning combined with in situ hybridisation provides good specificity and resolution for screening large numbers of plants in the required developmental stages, and allows reliable and statistically significant datasets to be
collected with a reasonable expenditure of time and effort. FISH and genomic in situ hybridization (GISH) are now well-established techniques for cytogenetical analyses and several groups have successfully applied these methods to wheat and its relatives (e.g. Schwarzacher et al. 1992). The physical size of introgressed segments can be assessed by GISH, which readily distinguishes between related genomes when total genomic DNA is used as a probe (Prieto et al. 2001), and the chromosomal distribution of repetitive sequences can be easily studied (Prieto et al. 2004a, 2004b).

We have used this protocol extensively to investigate plant nuclear organisation in cereals, which provide excellent models for studying chromosome behaviour in a range of tissue types. It provides a reliable tool to answer questions about the nature and timing of recognition events, chromosome associations and control mechanisms prior to and during meiosis (Martínez-Perez et al. 1999, 2000, 2001, Prieto et al. 2004b, 2004c), and to determine, for example, how universal meiotic events are in plants over a wide range of genome sizes and taxa (Martínez-Perez et al. 1999, 2000, 2001, Prieto et al. 2004b, 2004c).
MATERIALS

REAGENTS

- multi-well slides (ICN Biomedicals Inc.)
- Decon 90 (Decon Laboratories Limited)
- 3-aminopropyl triethoxy silane (APTES, Sigma)
- Acetone (Merck)
- glutaraldehyde (Merck)
- PBS buffer pH 7
- 100 mM DTT (Promega P117B)
- 100 mM dNTP (Roche)
- biotin-16-dUTP (Roche 11093070910)
- digoxigenin-11-dUTP (Roche 11093088910)
- DNApolI/DNAseI (Invitrogen 18162-016)
- 0.5M EDTA pH 8
- Minelute PCR purification kit (Quiagen 28004)
- Hybond N+ membrane (Amersham Biosciences RPN303B)
- 1M Tris-HCl pH 7.5
- 1M Tris-HCl pH 9.5
- 3M NaCl
- 1M MgCl₂
- Blocking reagent (Roche 1096176)
- Anti-digoxigenin-AP Fab fragment (Roche 11093274910)
- Anti-biotin-AP Fab fragment (Roche 1426303)
- 4-nitroblue-tetrazolium chloride (NBT, Roche 1383213)
- 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Roche 1383221)
- Fixative: 4% formaldehyde (w/v) in 1× PEM buffer (50mM PIPES/KOH pH 6, 5mM EGTA, 5 mM MgSO₄)
- Methanol: 30%, 50%, 70% and 100% (v/v)
- Cellulase (Onozuka R-10)
- Pectolyase (Kikkoman, Japan)
- 1× TBS buffer pH 7
- salmon sperm
- 20× SSC pH 7
- deionised formamide (Sigma F9037)
· Tween 20 (Sigma P1379)
· BSA (Sigma B8667)
· anti-digoxigenin antibody conjugated to FITC (Boehringer Mannheim Corp., Indianapolis, IN)
· extravidin conjugated to cy3 (Sigma S6402)
· DAPI
· Vectashield (Vector Laboratories)

EQUIPMENT
· Vibratome Series 1000plus (TAAB Laboratories Equipment Ltd., Aldermarston, UK)
· Tube heater, PCR machine or water bath (for labelling probes)
· Modified thermocycler (Omnislide; Hybayd LTD., Long Island, NY) for pre-treatment and in situ hybridization
· Water bath (for post-hybridization washing steps or for pre-treatment instead of the modified thermocycler)
· Humidity chamber set at 37ºC for probe detection (or in situ hybridization instead of the modified thermocycler)
· Confocal microscope equipped with suitable lasers (major providers: (Leica Microsystems, Carl Zeiss). For the best optical resolution use an oil-immersion objective of high NA (1.30) with a 63× or 100× magnification. Good results can also be obtained with high NA (1.25) water immersion objectives. For best optical results the refractive index of the specimen, of the specimen mounting medium and of the immersion medium at the objective should all be the same and should be correct for the objective; this is usually difficult to achieve completely.

REAGENT SETUP
**Dextran sulphate.** Dissolve 5 g dextran sulphate ultrapure MW500000 in TE buffer to a final volume of 10 ml by stirring and heating at 70ºC. Aliquots of the solution can be stored at -20ºC for years.

**NTB buffer.** For 500µl stock: 250µl 1M Tris-HCl pH 7.5; 25µl 1M MgCl₂; 125µl 20mg/ml BSA; 100µl water. It can be stored at -20ºC for years.

**Unlabelled dNTP mixture.** Mix 2µl of each 100mM dATP, dCTP, dGTP with 394 µl NTB buffer. It can be stored at -20ºC for months.
**Dot blot buffer 1.** Mix 10 ml 1M Tris-HCl pH 7.5; 5 ml 3M NaCl; sterile water up to 100 ml. It can be stored at room temperature for months.

**Dot blot buffer 2.** 0.5% (w/v) blocking reagent in buffer 1.

**Dot blot buffer 3.** Mix 10 ml 1M Tris-HCl pH 9.5; 1 ml 1M NaCl; 5 ml 1M MgCl₂; sterile water up to 100 ml. It can be stored at room temperature for months.

**Hybridization mix.** For 10 ml stock: 6.250 ml deionized formamide; 2.500 ml 50% dextran sulphate; 1.250 ml 20×SSC; 0.150 ml 10% SDS. Mix compounds well. Aliquots of the solution can be stored at -20°C for years.

**Blocking solution:** Add 0.15 g BSA and 0.2% (v/v) Tween 20 to 3 ml 4×SSC.
TIME TO ALLOW FOR FULL PROCEDURE

Pre-treatment of the slides (TIMING estimated 2 hours)

Fixation (TIMING 1 hour 30 min)

Sectioning (TIMING several days, depending on the number of samples. Usually sectioning one sample takes between 30 min and 1 hour)

Probe labelling (TIMING 2 hours 30 min)

Check the labelling reaction (TIMING 2 hours)

FISH: pre-hybridization steps (TIMING 2 hours)

FISH: hybridization (TIMING overnight)

FISH: post-hybridization steps and detection of the probes (TIMING 3 hours)

Image acquisition (TIMING estimated 1 hour per sample)
PROCEDURE

Pre-treatment of the slides
1| Wash the slides in 3% Decon for at least 1 hour and rinse them thoroughly with distilled water. Air-dry slides.
2| Coat the slides by dipping into a freshly prepared solution of 2% (v/v) APTES in acetone for 10 seconds.
3| Activate the slides with 2.5% (v/v) glutaraldehyde in phosphate buffer for 30 min, and rinsed in distilled water and air-dry slides.

Fixation
4| Cut the chosen plant material (root tips or florets in our case) and fix it in a 4% freshly made formaldehyde solution for 1 hour at room temperature. (Instructions for making formaldehyde?)
5| Wash samples in 1 TBS buffer for 10 min at room temperature.
PAUSE POINT. Fixed tissue can be stored at 4ºC up to 4 days but it is recommended to use it for sectioning within the same day.

Sectioning
6| Cut 20µm thick sections from root-tips or anthers using a vibratome and allowed to dry on multi-well slides.
7| Check the quality of the sectioned tissue by phase-contrast microscopy.
PAUSE POINT. Sectioned samples can be stored at 4ºC for several days. In some cases they can be stored with dessicant at -20ºC for several weeks.

Probe labelling
8| Mix: 5 µl 10× NTB buffer; 1 µl 100mM DTT; 5 µl unlabelled dNTP stock; 1 µl dUTP-biotin (or dUTP-digoxigenin); 2-3 µg DNA template (total genomic DNA, repetitive sequences amplified by PCR or BACs); 5 µl DNApolI/DNAsel; sterile water up to 50 µl final volume.
9| Incubate at 15ºC during 90 min in a water bath, tube heater or PCR machine.
10| Stop the labelling reaction by adding 5 µl 0.5M EDTA pH 8 and cold down on ice.
11| Purify the probe using the Qiaquick PCR purification kit (Alternatively probes can be purified by precipitation overnight with 100% ethanol at -20ºC).
PAUSE POINT. Labelled probes can be stored at -20ºC for months.
Check the labelling reaction (dot blot test)

12| Cut a piece of Hybond N+ membrane and soak in dot blot buffer 1 for 5 min. Air-
dry the membrane.
13| Load the membrane with 1 µl of the DNA probe and leave to air dry for 5-10 min.
14| Place the membrane in dot blot buffer 1 for 1 min.
15| Place the membrane in dot blot buffer 2 for 30 min shaking.
16| Incubate the membrane in (1:5000, v/v in dot blot buffer 1) anti-digoxigenin-AP or
(1:500, v/v in dot blot buffer 1) anti-biotin-AP solution for 30 min at 37°C.
17| Wash the membrane in dot blot buffer 1 for 15 min.
18| Wash the membrane in dot blot buffer 3 for 2 min.
19| Incubate the membrane in the dark in the detection buffer (prepare it freshly mixing
22.5 µl NTB; 17.5 µl BCIP; dot blot buffer 3 up to 5 ml) for the colour to develop.
20| Wash the membrane in water and leave to air dry.

FISH: pre-hybridization steps

21| Dehydrate slides in ascending methanol series (30%, 50%, 70% and 100%) 2 min
each and air-dry slides.
22| Incubate the tissue sections with the enzyme mix, 1% (w/v) cellulase, 0.5%
pectolyase (w/v) in TBS for 1 hour at 37°C in a humid chamber.
23| Wash the tissue sections in TBS for 10 min.
24| Dehydrate slides in ascending methanol series (30%, 50%, 70% and 100%) 2 min
each and air-dry slides.

FISH: hybridization

25| Add 1µl of (1mg/ml) salmon sperm and 1µl of labelled probe to the hybridization
mix stock.
26| Denature of the hybridization mixture at 95°C for 5 min., cool in ice for another 5
min. and immediately apply 20 µl (per well?) to the sections and put plastic coverslips
over them.
27| Denature slides and probe simultaneously in a modified thermocycler at 78°C for 8
min.
28| Cool down slides progressively in the thermocycler (50°C for 1 min, 45°C for 1 min,
40°C for 1 min and 38°C for 5 min).
29| Incubate slides in the thermocycler at 37°C overnight. Alternatively hybridization overnight can be done in a humid chamber.

FISH: post-hybridization steps and detection of the probes
30| Wash samples briefly at 42°C in 2× SSC to remove cover slips
31| Wash slides in 20% (v/v) deionised formamide in 0.1× SSC for 10 min at 42°C.
32| Wash slides in 2× SSC for 10 min at 42°C.
33| Wash slides in 2× SSC for 10 min at room temperature.
34| Wash slides in 0.2% (v/v) Tween 20 in 4× SSC for 10 min at room temperature.
35| Incubate samples with the blocking solution for 5 min at room temperature.
36| Incubate samples with an (1:100) anti-digoxigenin antibody conjugated to FITC or (1:500) extravidin-cy3 (for digoxigenin labelled probes or biotin labelled probes respectively) for 1 hour at 37°C in a humidity chamber.
37| Wash slides in 0.2% (v/v) Tween 20 in 4× SSC for 15 min at room temperature.
38| Incubate samples with 20 µl of (1µg/ml) DAPI for 10 min at room temperature in the dark.
39| Briefly wash slides in 0.2% (v/v) Tween 20 in 4× SSC for 10 min at room temperature.
40| Mount slides in Vectashield or similar anti-fading mounting medium adding 1 small drop on each well and add full-length glass cover slip.

PAUSE POINT. Mounted slides can be stored in a dark and dry place at room temperature for several days prior to image acquisition. (We store at 4°C?)

Image acquisition
41| Collect confocal optical section stacks. Our most recent publications have used a Leica TCS SP confocal microscope equipped with a Krypton and an Argon laser, or have used a conventional wide-field microscope with a cooled CCD camera followed by image deconvolution. The topic of image acquisition is a large one, and the reader is referred to the extensive literature on this topic (e.g. Handbook of Biological confocal microscopy).
42| In our work we have transferred the recorded data to NIH image (a public domain program for the Macintosh by W. Rasband available via ftp from ftp://Zippy.nimh.nih.gov). Images have been usually composed for publication using Adobe Photoshop 5.0 (Adobe systems Inc., Mountain View, CA).
CRITICAL STEPS

3] Activate slides just prior sectioning.
5] Vacuum infiltration is recommended to facilitate penetration of the fixative. Generally, good fixation is very important and a frequent source of problems.
7] Quality of the sectioned tissue is crucial in order to obtain good hybridization signals.
8] Quality of probes is critical for in situ hybridization. It is strongly recommended check the quality of the DNA in an electrophoresis gel prior to label it.
24] Do not store slides after the pre-hybridization treatment. Proceed with the in situ hybridization within hours.
26] Air-bubbles might hamper hybridization.
27] Over-denatured preparations will result in poor chromosome morphology and/or loss of tissue.
28] Cooling down probes and chromosomes gradually and simultaneously from the denaturation temperature to the hybridization temperature facilitates the annealing between the probe and the chromosomes, reduce background and also helps in the appropriate renaturation of the chromosome structure.
39] Do not allow slides to dry through steps 30-39.
40] The use of anti-fading reagent is very important for imaging of fluorescent dyes. The choice of a specific anti-fading depends on the label used. We found Vectashield the most suitable anti-fading reagent for FITC-labelled and Cy3-labelled DNA probes.
ANTICIPATED RESULTS

The use of the vibratome has been crucial for revealing detailed structures of internal plant cells that are not accessible to confocal microscopy on the intact plant or tissue. The thickness of sections that can be cut of unembedded plant tissue ranges from about 20µm to 1mm. Figure 1a shows a low magnification confocal image of a longitudinal section 1 mm thick along a wheat root showing the meristematic region. Apart from the caliptra and the apical meristem, all the internal tissues are visible and well-preserved, even the central column of developing xylem vessel cells.

Figure 1b shows a series of confocal images through a tranverse section through a wheat anther (anther or floret?). Each anther contains four locules, each of which comprises the central pollen mother cells (PMCs) or microsporocytes and the surrounding tapetal cell layers. This specimen is at an early stage of meiosis.

Tissue sections for FISH are usually between 20 and 50 µm thickness. ISH can be used on thicker sections but labelled probes do not penetrate efficiently to the innermost cells, and very thick specimens cause problems for high resolution microscopy, even using a confocal microscope.

FISH results, as for all molecular cytogenetics techniques, strongly depend on the quality of the tissue that is used. Preservation and morphology of the intact nuclei are especially important issues in achieving good FISH results.

Good penetration of the probes through the cell wall and into the nucleus is also very important when tissue sections are used for in situ hybridization experiments. Probe penetration is enhanced by dehydration in methanol and the treatment of the tissue sections with the cellulase and pectolyase enzyme mix, which partially digests cell walls. Probe penetration can be improved with a longer incubation with the enzyme mix, but a balance between digestion and structural preservation has to be found. Different plant species may require different cell wall digestion enzymes.

Some examples of different in situ hybridization experiments are presented here to illustrate the range of possibilities that this procedure has for investigation of plant nuclear organisation, using the protocol described.

A clear marker for the onset of meiosis is the presence of a telomere cluster in the PMCs (Martinez-Perez et al., 1999). At this point, PMCs display only one or two nucleoli (DAPI-negative circular regions in the nucleus) in anther development. Before telomeres form a cluster, PMCs and tapetal cells are not morphologically
distinguishable, and can be only classified by their position in the anther. To label telomeres we have used the telomeric sequence from *Arabidopsis thaliana* as a probe, amplified previously by PCR using the oligomer primers (5′-TTTAGGG-3′)_s and (5′-CCCTAAA-3′)_s in the absence of template DNA (Cox et al. 1993). Telomeres associated as a telomere cluster are shown in Fig 1c and e. A microsporocyte before telomeres form a cluster is shown in Fig 1f. In this meiotic stage, telomeres are still dispersed. For the unequivocal identification and staging of the cells, the general morphology of the flower, the number of nucleoli present in the nucleus, the morphology of the nucleus, the position of the cell in the anther, and the disposition of the telomeres can be used. This method has been successfully used to analyse chromosome association during early meiosis in rice, wheat and related species (Martínez-Pérez *et al.* 1999, 2000, 2001, Prieto *et al.* 2004b, 2004c).

This procedure not only enables repetitive DNA structures such as centromeres and telomeres and heterochromatin knobs to be labelled but also whole chromosomes or chromosome arms in nuclei containing DNA from more than one genome, as occurs in hybrids or in addition, translocation or substitution lines between related species. GISH has been previously used to assess chromosome association on intact nuclei (Martínez-Pérez *et al.*, 1999, 2001). We have used a wheat (*Triticum aestivum* cv. ‘Chinese Spring’) line carrying rye segments substituted for the equivalent region of the 1D pair of wheat chromosomes. These chromosomes still have wheat telomeres and sub-telomeric regions and the rye segment possesses a similar gene content but different repetitive content compared to the equivalent wheat region (Moore *et al.*, 1993). Thick anther sections were labelled simultaneously with total rye genomic DNA (green) and telomere probes (red) by *in situ* hybridization, and visualized by three-dimensional confocal microscopy (Fig 1c-f). The two rye DNA segments are observed as elongated structures when telomeres are clustering (Fig 1d, e). In the previous stage of meiosis the two rye segments appear as two foci (Fig 1f) as they do in somatic interphase nuclei.

Chromosome association has been investigated in cultivated rice (*Oryza sativa*, AA, 2n=24, cv. Bengal). *In situ* hybridization using the rice centromeric sequence (RCS2, GenBank Accession No. AF058902) as probe was used to detect centromeres in the interphase nuclei of intact root sections in *O. sativa*. Results are shown in Fig 1g. Centromeres remain largely unassociated in this cell type.

Bacterial artificial chromosomes (BACs) can also be detected by this protocol. A BAC for rice chromosome 1 was used as probe (Chen *et al.* 2002) for *in situ* hybridization.
The a0040G11 clone is located in contig 16 in rice chromosome 1 and mapped approximately at 100 cM (Chen et al. 2002). In situ hybridization with this BAC in roots tissue of *O. sativa* clearly shows two hybridization sites for each BAC corresponding to the two homologous chromosomes (Fig 1h).

The sequence pSc250, amplified by PCR using total rye genomic DNA as template to label rye heterochromatin knob DNA (Vershinin et al. 1995), and the *Arabidopsis* telomeric sequence were used for in situ hybridization on Chinese Spring/Secale cereale cv. Petkus F₁ hybrids. Results were visualized by three-dimensional confocal microscopy (Fig 1i). Telomeres are starting to cluster in one side of the nucleus (red) the heterochromatin knobs on each rye chromosome remain as tight foci (green).

Thus we have demonstrated that this procedure is suitable for analysing a wide range of probes and tissues. The examples that we show in this paper not only validate the procedure that we present but also show that in situ hybridization carried out in three-dimensional plant tissue is a powerful tool for investigating plant nuclear organisation.
REFERENCES


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ACKNOWLEDGEMENTS

This work was funded by the Biotechnology and Biological Sciences Research Council of the UK.
Figure 1
FIGURE/TABLE CAPTION

Figure 1. All images are projections from confocal sections spaced 0.5 μm.

a) Low magnification confocal image of a longitudinal root meristem vibratome section stained with DAPI. The caliptra and the apical meristem and all the internal tissues including the central column of developing xylem vessel cells are visible.

b) Low magnification confocal image of an anther vibratome section stained with DAPI. Transverse section through the anther including four locules with the microsporocytes in the central area.

c) Telomere behaviour during early meiosis in wheat. The projections have been produced to show the telomeres (red) in PMC at an early developmental stage. The telomeric probe was labelled with biotin and detected in red with anti-biotin-Cy3. Telomeres are associated as a cluster in one side of the cell.

d) Homologous segment behavior at the telomere cluster stage during early meiosis in wheat. Rye segments were labelled with digoxigenin and detected with anti-digoxigenin FITC (green segments).

e) Overlay of c and d.

f) Telomere behaviour during an earlier stage of meiosis. Telomeres have just started to associate to form the telomere cluster. The two rye chromosome segments are still decondensed and they can be seen as two foci.

g) Centromere behaviour on diploid rice. The rice centromeric probe was labelled with digoxigenin and detected with anti-digoxigenin FITC. Centromeres are associated in one side of the nucleus in the developing xylem vessel cells (bigger cells, arrowed) and they are dispersed on the nucleus in the rest of the root tissues.

h) Fluorescence in situ confocal images of a bacterial artificial chromosome (BAC) for rice chromosome 1 in root sections of diploid rice. The BAC was labelled with biotin and detected with anti-biotin-Cy3 in red.

i) Telomere and heterochromatin behavior in an early meiotic nucleus in the wheat-rye hybrid. Rye heterochromatin knobs are green and telomeres red.

Scale bar in a represents 100μm in a), 50 μm in b), 10 μm in c-e), g) and h) and 5 μm in f) and i).