Chapter 6

Immunolabeling and In Situ Labeling of Isolated Plant Interphase Nuclei

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Abstract

Specific labeling of proteins and nucleic acids by immunofluorescence or in situ techniques is an important adjunct to microscopical analysis for cell biology. Labeling of nuclear structures in intact complex tissues is often hampered by problems of penetration of the macromolecular labeling reagents needed. Here we describe a method of labeling isolated plant nuclei that we have found to be a useful approach that can help to overcome these problems.

Key words Immunolabeling, In situ hybridization, Plant cell biology, Nuclei, Nuclei isolation, Fluorescence microscopy

1 Introduction

Imaging of interphase nuclei by optical microscopy methods such as phase or differential interference contrast, or with fluorescence microscopy using a general DNA dye like DAPI, shows the overall shape and some substructural features, as, for example, nucleoli and heterochromatin. However, detailed imaging requires labeling of specific proteins or other components. Ideally, when analyzing living systems, it is best to image live organisms and cells by expressing proteins fused to tags such as GFP. But this is not always possible. Alternative methods are antibody labeling (immunofluorescence) or in situ hybridization to RNA or DNA sequences [1, 2] followed by fluorescent detection. These procedures require access of large molecules to the nuclear interior, which in turn requires opening up the structure to enable diffusion of these molecules; the resulting techniques are always a balance between efficiency of labeling and structural preservation of the specimens under investigation.

In multicellular plants, nuclei are located inside cells, which are surrounded with relatively impervious cell walls, and are embedded within tissues containing many cells and often multiple layers
of cells. This can make both the penetration of labeling reagents and the subsequent microscopy imaging either challenging or impossible. Often it is necessary to section the material before labeling (e.g., [3, 4]), or gently squashing it onto the slide. A very useful alternative is to image isolated nuclei. Here we describe methods for isolating plant nuclei as preparations on microscope slides or coverslips using a cytopsin centrifuge and labeling them by immunofluorescence or in situ hybridization. We have described these procedures for *Arabidopsis thaliana* seedling roots, but very similar methods can be used with minimal modification for other parts of the plant and for other species.

Two methods for releasing nuclei from roots are described below. Alternatively the simplest method is to repeatedly chop the roots with a sharp razor blade (not described). This is surprisingly effective for the small amounts needed for microscopy labeling. Once a technique for nuclei isolation has been established, the most common reason for failure is poor fixation. Formaldehyde is usually used for fixation of plant material for light microscopy. We advise that formaldehyde be freshly made from paraformaldehyde as described below, as it degrades in solution. Electron microscopy requires better fixation than formaldehyde can provide and glutaraldehyde is usually the fixative of choice. Small amounts of glutaraldehyde are sometimes added to formaldehyde for light microscopy, which can improve preservation, particularly for harsh treatments such as in situ labeling. But often the better preservation of cells prevents diffusion of the labeling probes into the specimen. Glutaraldehyde also causes a large degree of background fluorescence. This can be alleviated to some extent by treatment with sodium borohydride.

Some of the most informative studies of the nucleus involve the use of both immunofluorescence and in situ labeling on the same specimen, for example, to show the association of particular proteins with specific genes, or other DNA or RNA sequences. In these cases, it is generally best to carry out at least the primary antibody labeling before the in situ. This is presumably because the harsh denaturation conditions for in situ labeling destroy the antigenicity of the proteins, whereas the complexed antibody-antigen is extremely stable. However, each scenario is different and needs careful monitoring to determine the best sequence of operations. An example of a nuclear preparation labeled by two different immunofluorescence probes is shown in Fig. 1.

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## Materials

### 2.1 Plant Material Preparation

1. Sterile 9 cm square Petri dishes for plant growth media.
2. Plant growth medium: Murashige and Skoog (M&S) [0.025 mg/l CoCl₂·6H₂O, 0.025 mg/l CuSO₄·5H₂O, 36.7 mg/l Na Fe-EDTA, 6.2 mg/l H₃BO₃, 0.83 mg/l KI, 16.9 mg/l MnSO₄·2H₂O, 0.25 mg/l Na₂MoO₄·2H₂O,]
8.6 mg/l \( \text{ZnSO}_4 \cdot 7\text{H}_2\text{O} \), 332.02 mg/l \( \text{CaCl}_2 \cdot 2\text{H}_2\text{O} \), 170 mg/l \( \text{KH}_2\text{PO}_4 \), 1900 mg/l \( \text{KNO}_3 \), 180.5 mg/l \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), 1650 mg/l \( \text{NH}_4\cdot\text{NO}_3 \), pH 5.8].

3. 10% v/v bleach (store brands contains 5–10% sodium hypo-
chlorite) solution in \( \text{dH}_2\text{O} \).

4. \textit{Arabidopsis} seeds.

### 2.2 Cytotunnel Preparation

1. Microscope slides with frosted end.
2. Shandon Cytospin 4 Cytocentrifuge (Thermo Scientific).
4. Shandon Cytoclips™ (Thermo Scientific).

### 2.3 Immunofluorescence

1. Triton TX-100. Prepare a stock of 10% v/v Triton TX-100 in
\( \text{dH}_2\text{O} \) and store at 4 °C.
2. Dilute sulphuric acid. Prepare a solution of 10% v/v sulphuric
acid by the careful drop-wise addition of concentrated (98%)
sulphuric acid to \( \text{dH}_2\text{O} \).
3. The pH 4.5–10 indicator strips.
4. Phosphate buffer saline (PBS) pH 7.0 (for medium). 10× PBS
stock (10× PBS: 1.37 M \( \text{NaCl} \), 27 mM \( \text{KCl} \), 100 mM
\( \text{Na}_2\text{HPO}_4 \), 18 mM \( \text{KH}_2\text{PO}_4 \)).
5. Vacuum infiltration equipment. A plastic vacuum dessicator is
attached to a rotary vacuum pump. This equipment should be
situated in a fume hood.
6. Nuclei isolation buffer (NIB): 10 mM MES (2-(N-morpholino)
ethanesulfonic acid) pH 5.5, 0.2 mM sucrose, 2.5 mM EDTA,
2.5 mM DTT, 10 mM NaCl, 10 mM KCl, 0.1 mM Spermine, 0.5 mM spermidine, 0.5% Triton TX-100.

7. Flat-ended stainless steel rod (140 mm × 3 mm) and/or stainless steel grinder for 1.5 ml microcentrifuge tube (see Note 1 and Fig. 2).

8. Nylon mesh filter – either CellTrics disposable 30 μm filter (Partec) or homemade (see Note 2 and Fig. 3).

9. Blocking solution: 3% w/v bovine serum albumin (BSA) in PBS pH 7.0. Make fresh each time.

10. Homemade plastic coverslips made from transparent autoclave bags cut into the standard coverslip size (22 mm by 22 mm).

11. 4’, 6-Diamidino-2-phenylindole (DAPI) 1 μg/ml solution in dH2O. Protect from light and store at 4 °C.

12. 2, 2’-Thiodiethanol (TDE). 97% v/v TDE, 3% v/v PBS, pH 7.0. Store at 4 °C and protect from light.


15. Nail varnish/nail polish.

16. Glass embryo dishes (30 mm).

Fig. 2 Equipment for maceration as described in see Note 1. (a) Stainless steel rod with flat end. (b) Stainless steel grinder made to the internal profile of an Eppendorf tube (c)
2.4 In Situ Hybridization

1. Nucleic acid probes labeled with digoxigenin or biotin \((\text{see Note 3})\).
2. Formamide, deionized, minimum 99.5 \% \((\text{see Note 4})\).
3. Formamide, laboratory reagent grade.
4. Dextran sulfate.
5. 20× SSC (saline sodium citrate buffer). 3 M NaCl, 300 mM Tri-sodium citrate \((\text{Na}_3\text{C}_6\text{H}_5\text{O}_7)\) pH 7.0.
6. SDS (sodium dodecyl sulfate), preferably purchased as a 20 \% solution in dH₂O, to avoid handling the solid powder.
7. Salmon sperm DNA.
8. OmniSlide hybridization chamber \((\text{see Note 5})\).
10. Ethanol.

3 Methods

Carry out all procedures at room temperature unless otherwise stated.

3.1 Preparation of Plant Material

1. Prepare M&S media plates. Use Murashige and Skoog medium supplemented with 0.5 \% (w/v) Phytagel and 1 \% (w/v) sucrose; for 1 l of solution, autoclave for 20 min at 120 °C;
allow to cool to about 60 °C before pouring into 10 cm square Petri dishes while still molten under sterile conditions (see Note 6). Use approximately 60 ml solution per dish. Allow to cool and solidify before use.

2. Surface sterilize Arabidopsis thaliana seeds in 10% bleach for 10 min in 1.5 microcentrifuge tube, then wash with three changes of sterile water.

3. Plate out individual seeds at 2–3 mm spacing in two rows across prepared Petri dishes with M&S media, allowing space for root growth.

4. Stratify the seeds by incubating for 2 days at 4 °C in the dark (see Note 7).

5. Germinate and grow seedlings by placing plates vertically (see Note 8) in a 25 °C growth chamber under constant illumination (see Note 9). Use approximately 5-day-old seedlings for the preparation of nuclei. Older plants can be used to enrich for endoreduplicated nuclei, whereas younger plants can be used to enrich for diploid nuclei (such as in meristematic cells). Also see Note 10 on glasshouse-grown plants.

### 3.2 Preparation of Fixative

Prepare an 8% w/v solution using prilled paraformaldehyde (see Note 11). Make the solution by adding paraformaldehyde to dH₂O on a heated stirrer in a fume cupboard (see Note 11). Warm to approximately 60 °C and make alkaline by the addition of a few drops of 1 M NaOH. The paraformaldehyde should dissolve to give a clear solution of 8% formaldehyde. Immediately prepare a solution of 4% formaldehyde in PBS by adding an equal volume of 2× PBS pH 7.0 to the 8% formaldehyde solution. This will give a final concentration of 4% formaldehyde in 1× PBS (see Note 11). Adjust the pH to 7 using dilute H₂SO₄ (see Note 12). Add Triton TX-100 to 0.01%.

### 3.3 Assembly of Cytofunnel Unit

1. Place a plain glass slide with frosted end into the cytoclip, keeping the frosted end to the outside of the clip.

2. Position a single white cytofunnel over the slide and secure with the cytoclip. Label appropriately. Figure 4 shows the cytofunnel components separately (Fig. 4a, b, and c) and assembled ready to load into the cytopin centrifuge (Fig. 4d).

### 3.4 Immunofluorescence Procedure

1. Cut root tips (up to 10 mm in length) (see Note 13) from Arabidopsis seedlings while still on plates. Collect 50–100 root tips and place into 20 ml of fixative in a 30 ml glass bottle.

2. Vacuum infiltration of fixative. Place the open glass bottle containing the fixative and tissue samples in the vacuum dessicator and replace the dessicator lid. Switch on the vacuum pump and
open the vacuum valve carefully to slowly pump out the air until the fixative solution bubbles gently. After about 5 min, release the vacuum and see if the tissue sinks in the fixative solution. If it still floats, repeat the vacuum procedure.

3. Incubate in the fixative for 1 h.

4. Wash roots in PBS pH 7.0 for 10 min, repeat twice.

5. Place washed roots into 300–400 μl of nuclei isolation buffer (NIB) in a 30 mm glass embryo dish and macerate roots vigorously with a flat-ended stainless steel rod (see Note 14). Continue macerating for several minutes until the roots have been reduced to tiny pieces releasing the nuclei into the NIB. Alternatively nuclei can be extracted by placing the fixed roots into a 1.5 ml microcentrifuge tube with the NIB, and then a stainless steel grinder can be used to grind the material to release the nuclei.

6. Filter the nuclei solution through a 30 μm nylon mesh filter (see Note 15).

7. Pipette 50 μl of the filtered nuclei into each assembled cytofunnel and spin in the cytofunnel at 500 rpm (30 × g) for 3 min.

8. Disassemble the cytofunnel units, remove the slides, and allow them to air-dry for 40–50 min.
9. Immerse the slides in 70% ethanol for 30 min.
10. Wash with PBS, pH 7.0 for 10 min, repeat twice.
11. Block tissue with 3% BSA in PBS pH 7.0 for 1 h (see Note 16).
12. Apply primary antibodies diluted appropriately in blocking solution (3% BSA in PBS) and incubate for a minimum of 2 h at room temperature, or up to a maximum of overnight at 4 °C. It is important to avoid drying of the solutions on the slides; place incubating slides in a sealed container such as a plastic Petri dish along with moist filter paper and/or use plastic autoclave bag coverslips.
13. Wash with PBS, pH 7.0 for 10 min, repeat five times.
14. Apply appropriate secondary antibodies diluted in blocking solution and incubate for 2 h at room temperature.
15. Wash with PBS, pH 7.0 for 10 min, repeat five times.
16. Counterstain for DNA with a 1 μg/ml solution of DAPI in H₂O for 30 min.
17. Wash with PBS, for 10 min, repeat once.
18. Remove as much liquid as possible and add 10–15 μl of a suitable mounting medium (see Note 17) and cover with a glass cover slip (see Note 18).
19. Seal the coverslip to the slide with nail varnish.
20. View samples with a suitable microscope.

3.5 In Situ Hybridization Procedure

1. Follow procedure for immunofluorescence to step 9 of Subheading 3.4.
2. Immerse slides in 100% ethanol for 10 min and then allow to air-dry.
3. Apply 25 μl of a hybridization mixture (7 ng/μl labeled DNA, 50% formamide, 10% dextran sulfate, 2× SSC, 0.125% SDS, 1 μg/μl salmon sperm DNA) to the slides and cover with a plastic coverslip.
4. Denature the probes and tissue simultaneously at 75 °C for 10 min and allow hybridization to proceed at 37 °C in a humid chamber for at least 16 h (see Note 5).
5. Following hybridization, remove the coverslips and wash the slides for 5 min successively in 2× SSC at 42 °C, then twice in 20% formamide, 0.1× SSC at 42 °C, twice in 2× SSC at 42 °C, twice in 2× SSC at room temperature, twice in 4× SSC/0.2% Tween 20 at room temperature, and finally in PBS pH 7.0.
6. Incubate the slides in blocking solution for 1 h.
7. Apply an appropriate fluorescently labeled antibody for digoxigenin or fluorescent labeled avidin/streptavidin/extravidin for
biotin, diluted in blocking solution, to the slides, and incubate for 2 h.

8. Continue from step 13 of the immunofluorescence procedure of Subheading 3.4 to end.

1. Follow the Immunofluorescence procedure of Subheading 3.4 to step 13.

2. Fix in 4% paraformaldehyde for 10 min at room temperature (optional) (see Note 19).

3. Wash in PBS, pH 7.0 for 10 min, repeat twice.

4. Follow the in situ hybridization procedure of Subheading 3.5 from steps 3–6.

5. Apply fluorescently labeled secondary antibodies to detect the probe labels (digoxigenin or biotin) and to recognize the primary antibodies used for the immunofluorescence. The secondary antibodies should be applied diluted in blocking buffer and incubated for 2 h.

6. Follow steps 15–20 of the immunofluorescence procedure of Subheading 3.4.

4 Notes

1. The stainless steel maceration rod was made by cutting a 140 mm length of 3 mm diameter rod and removing any sharp edges by gently grinding the cut edges to leave a flat-bottomed rod (Fig. 2a). The grinder fitting 1.5 microcentrifuge tube was turned from a 20 mm × 8 mm piece of stainless steel rod to give the internal shape of an Eppendorf tube (10° angle) with the end rounded to fit the bottom of the tube. This was screw tapped to accept the 5 mm stem also made from stainless steel that is screwed into the head. A plastic handle can be added for comfort (Fig. 2b, c).

2. A homemade filter can be made from the body of a 20 ml syringe with the tip cutoff. The cut end is then covered with a piece of 30 μm nylon net filter (Millipore) and secured with tape (Fig. 3).

3. Make nucleic acid probes using standard techniques to incorporate tags, which can subsequently be detected by relevant antibodies [1, 2]. The technique described in this protocol is for detection of DNA probes but a similar procedure can be followed for the detection of RNA probes.

4. A high purity grade of formamide should be used in the hybridization mixture, but laboratory reagent grade is sufficient for the washing solutions.
5. We used a Hybaid OmniSlide Thermal Cycler for the hybridization step. This has a built in humidity chamber that prevents drying out of the hybridization mixture on the slides. We used a program with an initial denaturation step of 75 °C followed by a gradual ramp down of the temperature to a final hybridization temperature of 37 °C, which was then held for at least 16 h. Unfortunately, this Thermal Cycler is no longer commercially available, although other models are available. However, a humidity chamber can easily be made from a plastic box with a fitted lid lined with wet tissue paper and a sheet of plastic to lay the slides on. Slides can be denatured on a flat bed heat block, then quickly transferred to the humid chamber and incubated at 37 °C.

6. It is not possible to remelt Phytagel, so allow the medium to cool to a reasonable working temperature of about 60 °C before pouring the plates under sterile conditions.

7. Stratification of seeds by a cold treatment of 2 days will ensure an even germination rate.

8. By placing the plates vertically, the germinating roots will grow along the surface of the gel and can be removed easily without damage to the root structure.

9. We use 25 °C, constant light growth conditions to germinate Arabidopsis seedlings. However, this procedure works equally well with seedlings grown at cooler temperatures with light/dark cycles. It should be noted that different conditions need different times to reach the same stage of development.

10. Although healthy looking plants can be grown year round in today’s glasshouses and controlled environment rooms, it is a frequent observation, although we have never seen documented, that the best immunolabeling or in situ labeling is obtained with plants grown close to their native growing season. For example, wheat in the UK gives the best labeling results when grown between April and October, even in glasshouses. In southern Spain, wheat produces the best results in the winter and very poor results in the hot summers. We are unsure of the reason for this; it is likely that even the best controlled environment rooms cannot completely remove the influence of the external environment, and this stresses the plants to some extent, even though they look healthy. This in turn could influence the production and composition of such components as the cell walls, which may make penetration of labeling reagents more difficult.

11. We find it best to make fresh formaldehyde each time. Formaldehyde may cause cancer so should always be used in a fume hood. Weigh out paraformaldehyde in the fume hood, wearing appropriate safety clothing, lab coat, gloves, and eye
protection. Warm the solution but do not allow it to boil, as this will degrade the formaldehyde. We recommend using paraformaldehyde “prilled” rather than powder to avoid harmful dust. Paraformaldehyde dissolves best at alkaline pH. Therefore it is best to make it in dH$_2$O with a few drops of alkali (rather than in a buffer) and then add a 2× buffer once the formaldehyde is dissolved. In this manner, less acid is needed to bring the pH back to neutral. If more than a ten drops of alkali per 100 ml of liquid are needed to dissolve the paraformaldehyde, this is probably a sign that the paraformaldehyde has degraded and should be replaced. Paraformaldehyde should be kept dry at all times. It lasts longer when stored at 4 °C in the dark than at room temperature, but should be allowed to warm to room temperature before opening the container to avoid condensation.

12. To adjust the pH of formaldehyde, do NOT use HCl, as reaction of formaldehyde and HCl produces the carcinogen Bis (chloromethyl) ether. Use pH strips to determine the pH rather than a pH electrode, as fixatives can degrade pH electrodes.

13. Using the first 10 mm of the root tip will ensure the availability of nuclei from both meristematic and differentiated tissue.

14. The maceration step can take several minutes of continuous stabbing with the flattened tip of the stainless steel rod to effectively release a substantial amount of nuclei. A good guide is to reach a point when there are very small pieces of root remaining and the solution is partially cloudy.

15. The nylon mesh filter should be wetted with NIB prior to use.

16. To ensure the blocking and labeling solutions keep in contact with the sample, either a plastic coverslip can be placed over the sample and solution, or a temporary well can be made by using a PAP pen to draw a well around the sample area. For in situ hybridization, the plastic coverslip should be made from a heat-resistant plastic such as an autoclave bag so that it is resistant to the high denaturation temperatures used.

17. There are many mounting solutions available, but it is important for optimal image collection to match refractive indexes as closely as possible within your imaging setup. Ideally the refractive index of the immersion medium for the lens (in this case oil), the glass coverslip, and the sample mounting medium should be the same. The mounting medium should also have good anti-fade properties and be able to limit the amount of fluorescence quenching through photobleaching. We have found that a solution of 97% TDE in PBS pH 7.0 [6] is very good for cy3 and cy5 and other fluorochromes at these longer wavelengths. However the fluorescence of Alexa 488 is less stable and Vectashield is better for this fluorochrome.
18. Most objectives designed for use in high-resolution biological imaging are calculated for a coverslip thickness of 170 nm (No 1.5). For the highest quality imaging, we recommend using high-precision coverslips such as Carl Zeiss high-performance coverslips, as these have a much smaller deviation from the nominal 170 nm than standard coverslips.

19. An extra fixation step is sometimes included if the antigen/antibody complex is suspected to be unstable and could be disturbed by the conditions of the in situ hybridization. Over-fixation, however, can lead to penetration problems of probes and antibodies, so careful monitoring of this step is important.

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References