

TECHNICAL ADVANCE

3D gold *in situ* labelling in the EM

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Summary

We have developed a novel pre-embedding *in situ* hybridization labelling method for electron microscopy which has given much greater sensitivity and higher labelling levels than have been achieved previously, together with good ultrastructural preservation. Vibratome sections of plant tissue were labelled throughout their thickness with 1 nm gold antibodies and then silver enhanced, embedded in resin and sectioned for electron microscopy. Because the labelling extends throughout the depth of the specimen, this method permits the study of the 3D arrangement of the labelling at the electron microscope level by either stereo-pair recording, tomographic reconstruction or 3D reconstruction from serial sections. In this paper we describe the application of this method to study the organization of rDNA in pea root tissue.

Keywords: 1 nm immunogold, 3D electron microscopy, rDNA, *in situ* hybridization, plant, nucleolus.

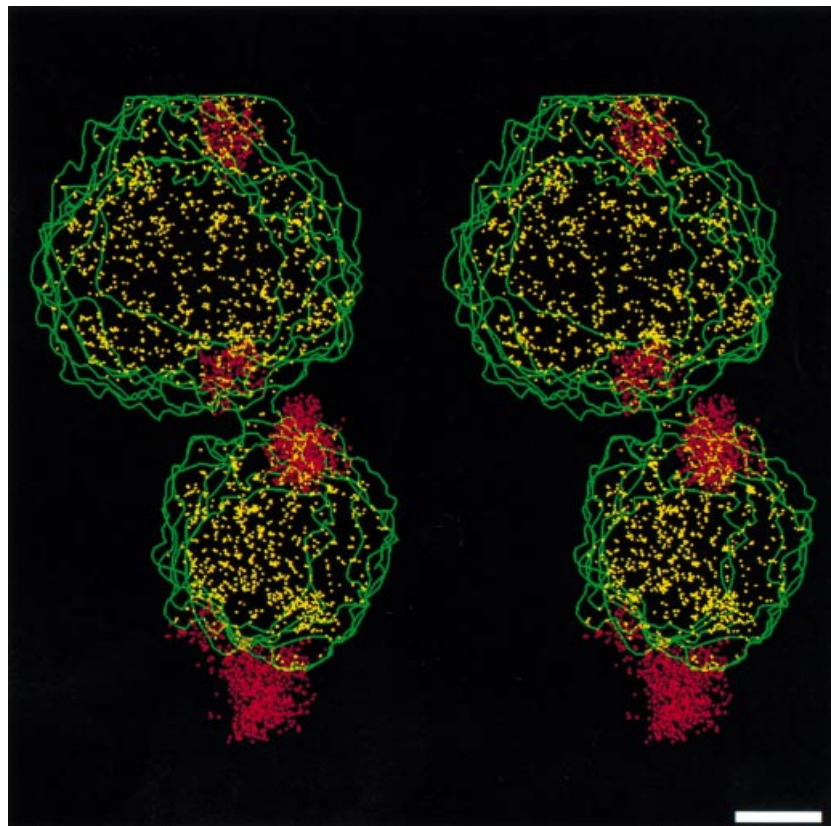
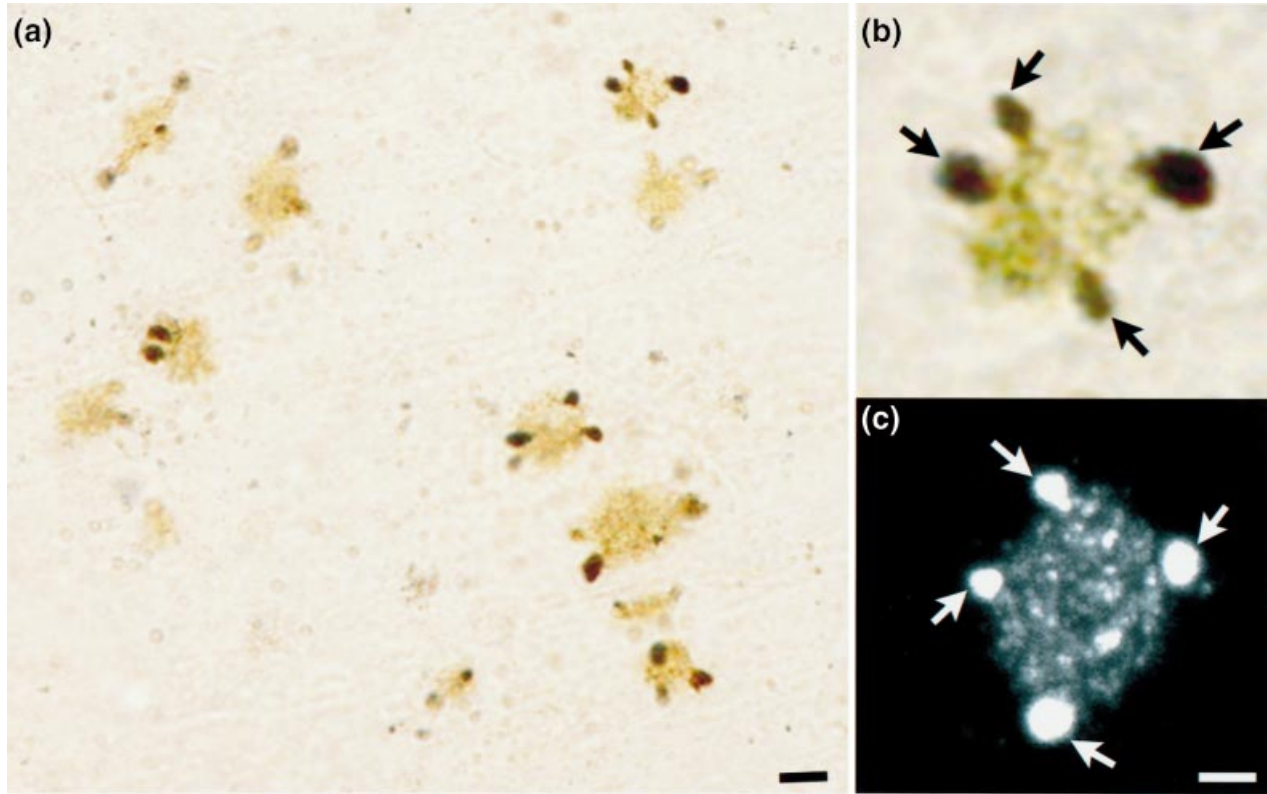
Introduction

The completion of several entire genome sequences, and the availability of genomic resources such as ordered BAC libraries, means that probes are readily available for virtually any gene or other DNA sequence of interest in several organisms, including *Arabidopsis* and rice, as well as other plant species. Thus, in principle, these genomic resources should allow a detailed analysis of the structural organization of the genome and its activities within the nucleus. To date, however, most structural genomic analysis has been carried out using various types of *in situ* fluorescence optical microscopy, largely because of the technical difficulties of labelling studies in the electron microscope (EM). However, it is clear that many questions of genomic and nuclear organization will require the higher resolution available to the EM. We present here a technique that will be useful for extending 3D analysis using *in situ* hybridization to electron microscopy.

Conventional EM *in situ* labelling, like EM antibody labelling, is carried out using colloidal gold probes on ultrathin sections from resin-embedded specimens (reviewed by Skeper, 2000). This technique provides very good preservation of the cell ultrastructure, and is useful for determining structure–function relationships by assigning gold particles to particular cell compartments and

organelles. However, although an EM section is a 3D object, and 3D information can be recovered either by collecting stereo views or by tilted-view tomographic reconstruction, only targets at the surface of the sections are accessible to the gold probes, and the third dimension is lost to the labelling. This leads to two further problems. First, the intensity of labelling is low, as only a small fraction of the potential labelled targets are accessible to the labelling reagents. Second, it is difficult to assign gold particles unambiguously to particular structures, as what is seen in a single electron micrograph (effectively a projection) is not the same as what is accessible to the probe.

These problems can be overcome at the optical level of resolution by using methods that allow the labelling to extend throughout the depth of individual cells or tissue sections, followed by confocal or other types of 3D microscopy (Aragon-Alcaide *et al.*, 1998; Thompson *et al.*, 1997). Correlative approaches, involving simultaneous detection with fluorochromes and 6 nm gold particles, have been successfully applied to image the same structures at both optical and the electron microscope levels on 100–200 nm cryosections of animal cells in culture (Pombo *et al.*, 1999). However, these methods would require serial



cryosectioning to reconstruct an entire cell or nucleus – technically an extremely difficult feat which has not so far been achieved. They also require specialized equipment, which is not widely available.

In this paper we present a novel, straightforward labelling technique for 3D EM in plant tissues. This method does not require expensive equipment or a state-of-the-art electron microscope, and can be used for both immunogold labelling in the nucleus and *in situ* hybridization. The method uses pre-embedding labelling with 1 nm gold antibodies, which are sufficiently small to penetrate through thick sections of plant tissues, and subsequent silver enhancing. This has given much greater sensitivity and higher labelling levels than have been achieved previously, together with good structural preservation. 3D data have been obtained by collecting, stacking up and modelling serial sections, or by recording stereo-pairs. This method is also very well suited to 3D reconstruction by computed tomography (Heliot *et al.*, 1997). We have used a similar method to determine that nucleolar transcription sites represent individual transcribed rDNA genes, which are organized as linear, compacted 'Christmas trees' (Gonzalez-Melendi *et al.*, 2001). Here we use this method to demonstrate the 3D organization of rDNA in pea root nucleoli by EM *in situ* hybridization.

Results and discussion

In situ hybridization was performed in 4% formaldehyde-fixed 40 µm vibratome sections of pea roots on microscope slides, and the hybridized probe was then detected by silver-enhanced 1 nm-gold antibodies. The resulting silver-gold label can be imaged by both bright-field light microscopy and transmission electron microscopy, facilitating correlative light and electron microscopy. For this study we used digoxigenin-labelled sense RNA probes for two different regions of the rDNA repeat – NTS is the intergenic spacer region (2.2 kb) and ITS1 is the internal spacer between the 18S and 5.8S transcribed regions (≈200 bases). Figure 1 shows a typical light microscopy image of a vibratome section labelled with the NTS probe. The silver-gold labelling seen in Figure 1(a,b) is comparable to that seen using confocal fluorescence microscopy on sections labelled by fluorescence *in situ* hybridization (Figure 1c). The highest intensity of labelling was observed

on four knobs (large arrows) of extra-nucleolar rDNA, corresponding to inactive copies (Thompson *et al.*, 1997). Within the nucleolus, a pattern of labelling consisting of threads and foci was observed.

As with all such labelling techniques, particularly *in situ* methods, a compromise had to be reached between the accessibility of the gold probes to their target, across the thick plant cell walls, and good structural preservation. Although post-section EM *in situ* hybridization is compatible with glutaraldehyde fixation, this fixative highly cross-links the tissue and prevents the penetration of the gold probes through the tissue. Another critical step was permeabilization. We found that dehydration into methanol and subsequent rehydration, coupled with cellulase treatment, was necessary to permeabilize the cells. Finally, the intensity of labelling was enhanced by a mild treatment with pronase.

The labelled vibratome sections were then processed, embedded in resin and sectioned for TEM using standard methods. The vibratome sections were labelled throughout their thickness, as the signal was seen on EM sections cut from either the top of the vibratome section (exposed to the labelling reagents) or the bottom (attached to the slide and thus least accessible). Although a somewhat higher intensity of labelling was observed on the top-most sections, no difference was seen in labelling intensity across an individual nucleus. An acceptable ultrastructural preservation of the cell nucleus was observed using conventional heavy metal counter-staining; the level of detail seen was comparable to that seen with conventional EM sections. Figure 2 shows a series of 0.5 µm sections through a single nucleus. Despite the relatively large thickness for EM, we were able to obtain excellent images on a conventional EM at 80 kV. Six out of the seven sections containing the two nucleoli in this nucleus are shown in Figure 2. The nuclear chromatin (chr) was seen as regions of low electron density separated by more electron-dense interchromatin regions (ir). Considerable substructure was visible in the chromatin regions. This pattern of negative chromatin staining is similar to that given by the EDTA cytochemical method for ribonucleoprotein (RNP)-containing structures (Bernhard, 1969). The nucleolus (nu) also showed considerable substructure, containing two differently stained regions, medium electron density at the periphery and pale in central regions.

Figure 1. *In situ* hybridization with a probe to the non-transcribed spacer (NTS) of the rDNA repeat from pea. Unembedded pea root vibratome sections. (a,b) 1 nm gold-label silver-enhanced and observed with bright-field light microscopy.

(c) For comparison, fluorescence *in situ* hybridization on another specimen is shown after confocal imaging. The highest intensity of labelling is seen in the four perinucleolar knobs of condensed rDNA (arrows). The intranucleolar labelling consists of threads and foci. The nuclear and cytoplasmic background is very low. Scale bars: 5 µm (a); 1 µm (b,c).

Figure 3. Stereo pair of the 3D model of the labelling from the serial section series shown in Figure 2, made using IMOD.

The nucleolus is outlined in green, the particles in the perinucleolar knobs are marked by red dots, and the internal nucleolar labelling by yellow dots. Scale bar, 0.5 µm.

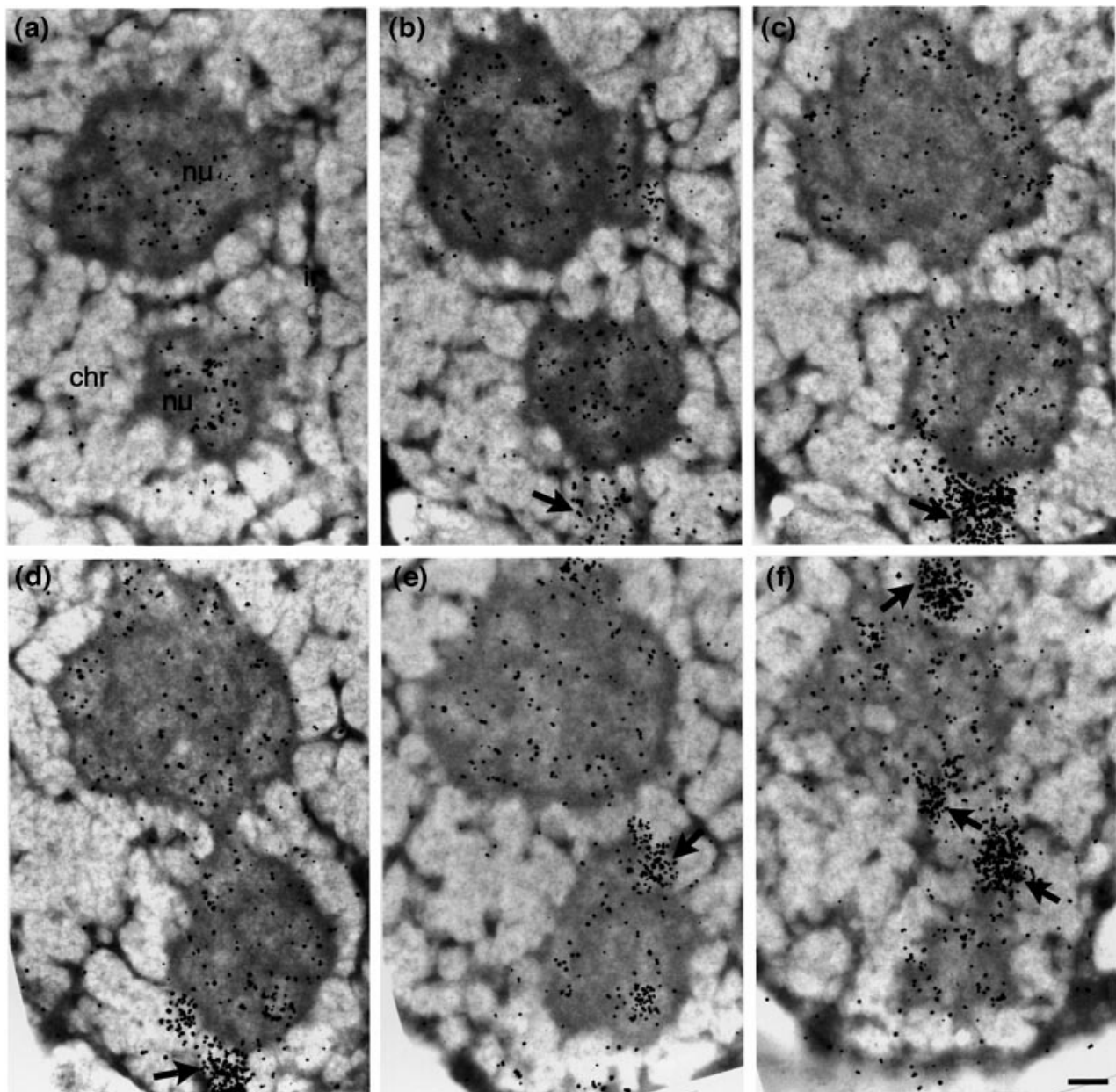


Figure 2. Series of six out of the seven 0.5 μm serial EM sections containing the two entire nucleoli of a single cell. *In situ* hybridization with a probe to ITS1.

The general nuclear morphology is well preserved. The nuclear chromatin (chr) is seen as regions of low electron density separated by more electron-dense interchromatin regions (ir). The nucleolus (nu) contains two differently stained regions, medium electron density at the periphery and pale in central regions. The perinucleolar labelling is seen as highly packed particles in the knobs (arrows). Within the nucleolus, the labelling is seen as single particles or pairs of particles predominantly in the pale regions. Scale bar, 0.5 μm .

The pale regions correspond to the dense fibrillar component – proteinase K treatment on thin sections has previously been shown to result in a low level of staining to this nucleolar component (Gonzalez-Melendi *et al.*, 1998).

In order to combine serial sections such as those shown in Figure 2 into a 3D reconstruction of the complete nucleoli, we used computer-aided modelling (IMOD;

Kremer *et al.*, 1996). Figure 3 shows a stereo-pair of the 3D reconstruction from this series in which the nucleolus is outlined in green, the particles in the perinucleolar knobs are marked in red, and the internal nucleolar labelling is coloured in yellow. Sections of this thickness (0.5 μm) contain considerable 3D information, which is lost in a single view. We used stereo pairs to interpret the 3D structure within individual sections. These sections would

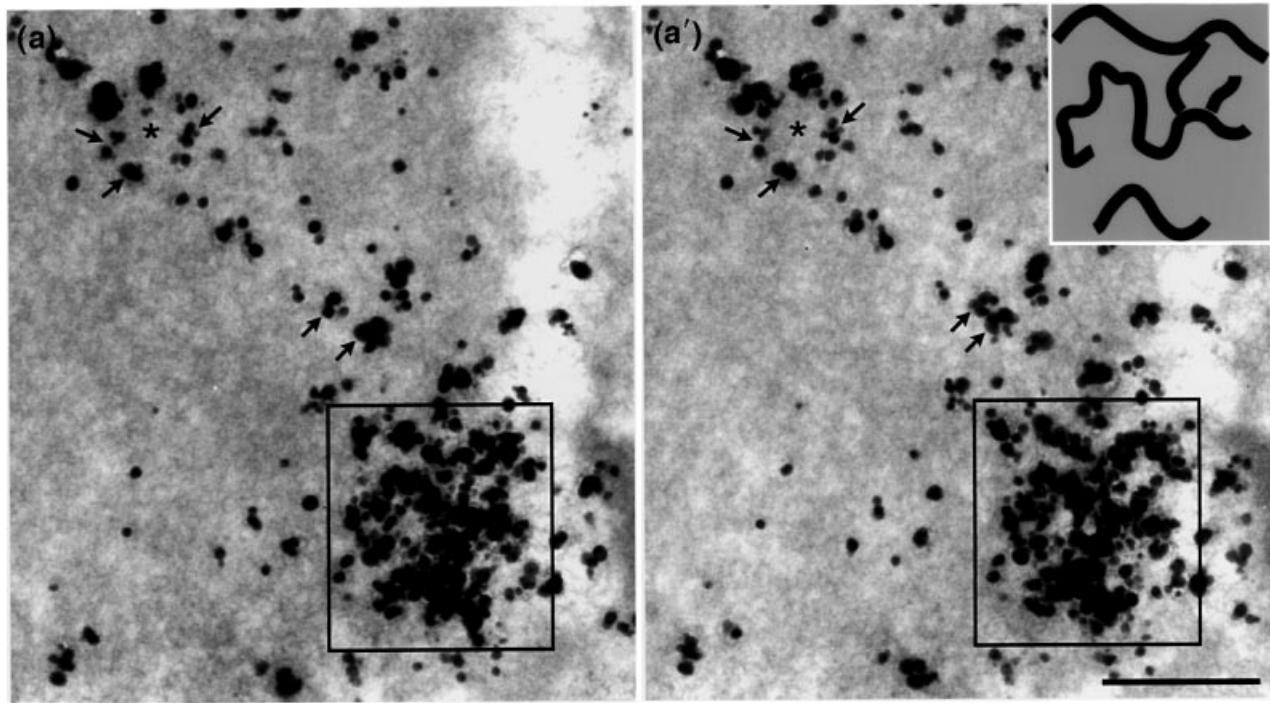


Figure 4. Stereo EM pair of a small region of a single nucleolus showing details of labelling by NTS *in situ* hybridization. Three-dimensional data are obtained in this case by tilting a single thick (0.5 μm) section in the EM. Part of one perinucleolar knob and some internal nucleolar labelling is present in this image. In the knob, the particles are organized in lines (boxed areas and inset diagram). Within the nucleolus, a more dispersed pattern of labelling was observed. There were frequent examples of clusters containing about four particles (arrows). Occasionally, clusters were grouped around an unlabelled central region (asterisk). Scale bar, 0.5 μm .

be eminently suitable for tomographic, tilted view reconstruction, which could also be carried using IMOD or one of several other packages available.

Using stereo-pairs, we observed that in the knobs the particles were often organized in lines (boxed areas and inset in Figure 4). This must correspond to the path of the condensed chromatin. Highett *et al.* (1993) previously detected substructure within the perinucleolar knobs, based on deconvolution of confocal images. We can now see these structures in much greater detail at the EM level. Within the nucleolus, a more dispersed pattern of labelling was observed, predominantly in the pale regions, with both NTS and ITS1 probes (Figures 2 and 4). With the NTS probe, there were frequent examples of clusters containing four or more particles (arrows in Figure 4). ITS1 labelling was generally represented by either single particles, or occasionally by pairs of particles (Figure 2). The different level of labelling seen using probes to the NTS and the ITS1 sequences can be related to the different size of the two target regions probed (2.2 kb and ≈ 200 bases, respectively).

In the reconstruction of the entire nucleoli using ITS1 shown in Figure 3, we counted approximately 1800 particles inside the nucleoli and about 1400 particles in the knobs, and other reconstructions showed similar

numbers. The total number of particles is thus comparable to the number of rDNA repeats in this species (approximately 4000), and this corresponds to an average of one particle per ITS1 repeat. This proportion of rDNA repeats in the nucleolus and in the perinucleolar knobs is consistent with previous measurements of integrated fluorescence intensity using confocal fluorescence *in situ* hybridization (Highett *et al.*, 1993). Previously we have shown about 300 active rDNA genes in these nuclei (Gonzalez-Melendi *et al.*, 2001), and the present data therefore suggest that there are many inactive rDNA repeats within the nucleolus, probably several times as many as the actively transcribed repeats.

In summary, we have developed a pre-embedding *in situ* labelling method for plant tissues that can bring together data from light and electron microscopy and can be used to study the 3D arrangement of specific gene sequences at the EM level. Although we demonstrate the method on pea root tissue, it should be well suited to any tissue which can be vibratome-sectioned, and which allows penetration of the labelling reagents. In our laboratory, equivalent fluorescence labelling has been obtained on vibratome sections of root and floral tissues of many different species (including maize, wheat and other cereals, *Arabidopsis*

and soyabean), although we have not so far used silver-gold detection in these systems. Using this approach, the level of labelling is much higher than in conventional post-section labelling, largely because the whole depth of the section is accessible to the labelling reagents. The level of labelling we have obtained should allow the detection of single-copy sequences such as BAC probes or single genes, which would be extremely unlikely to be found at the accessible surface of an ultrathin resin section, and would be therefore virtually impossible to label by conventional EM *in situ* methods. Thus, in principle, this method should allow structural analysis of the genome to be extended to the electron microscope.

Experimental procedures

Materials

Seeds of *Pisum sativum* L. (pea) cv. Alaska were imbibed in aerated water for 12 h and germinated at 18°C for 2 days on water-soaked tissue paper.

Preparation of probes

Non-transcribed spacer (NTS) probe. Digoxigenin-labelled sense RNA probes to the NTS from pea were synthesized by *in vitro* transcription as previously described (Thompson *et al.*, 1997).

Internal transcribed spacer 1 (ITS1) probe. Digoxigenin-labelled sense RNA probes to ITS1 from pea were synthesized by *in vitro* transcription as previously described (Beven *et al.*, 1996). Probe size was reduced to approximately 100 bases by a mild carbonate hydrolysis (Cox *et al.*, 1984).

In situ hybridization in tissue sections

The root tips were fixed in 4% formaldehyde in PEM buffer (PEM: 50 mM PIPES/KOH pH 6.9; 5 mM EGTA; 5 mM MgSO₄ pH 7.4) for 1 h at room temperature. After washing in PBS (PBS: 140 mM NaCl; 3 mM KCl; 4 mM Na₂HPO₄; 2 mM KH₂PO₄ pH 7.4), 40 µm vibratome sections (Vibratome series 1000, TAAB Laboratories Equipment Ltd, Aldermaston, UK) were cut under water and dried down on APTEs-coated multi-well slides. The sections were dehydrated in a series of 30, 50, 70 and 100% methanol/water, then rehydrated in a series of 70, 50, 30% methanol/water, and finally in PBS, for 5 min at each step. To facilitate penetration of the labelling reagents, the sections were treated with 2% (w/v) cellulase (Onozuka R-10) in PBS for 1 h at room temperature in a humid chamber, 0.125 mg ml⁻¹ pronase (Sigma Aldrich, Poole, Dorset, UK) (pronase buffer: 50 mM Tris-HCl, 5 mM EDTA pH 7.5) for 15 min at 37°C and 0.1% Tween 20 in PBS for 10 min at room temperature. After each treatment, the sections were washed with PBS for 10 min. The sections were allowed to dry and the hybridization mixture was added. The hybridization mixture contained: ≈200 ng µl⁻¹ digoxigenin-labelled probe; ≈1000 ng µl⁻¹ unlabelled RNA transcribed from a plasmid containing an unrelated insert; 50% de-ionized formamide; 10% dextran sulphate; 300 mM NaCl; 10 mM PIPES pH 8.0; 1 mM EDTA. The slides were

transferred to a modified thermocycler (Omnislide, Hybaid Ltd, Long Island, NY, USA) where target DNA denaturation was performed at 78°C for 10 min. Hybridization was carried out overnight at 37°C in a humid chamber. Then the sections were washed in 0.1 × SSC (SSC: 150 mM NaCl; 15 mM sodium citrate) for 90 min at 50°C.

Pre-embedding immunogold labelling

Detection of *in situ* hybridization was performed by incubating the sections with mouse anti-digoxigenin antibodies (Sigma) applied 1/5000 in 3% BSA in PBS for 90 min at room temperature. A secondary anti-mouse 1 nm gold antibody (British BioCell International, Golden Gate, Ty Glas Avenue, Cardiff, UK) was applied 1/100 in PBS overnight at 4°C. (The inclusion of BSA as a blocking agent in this solution was not found to be necessary, as good labelling with low background was obtained without it.) After extensive washes in PBS and water, the gold particles were amplified using a silver-enhancing kit (British BioCell International). The reaction was monitored under an inverted light microscope. When a faint signal was detected, silver enhancing was stopped in water. Then the sections were post-fixed with 1% glutaraldehyde in PBS for 15 min and washed twice in PBS for 10 min each before processing for electron microscopy.

Fluorescent labelling and confocal microscopy

Vibratome sections were hybridized with probes to NTS and ITS1 as described above. Then the hybridization signal was detected by mouse anti-digoxigenin antibodies (Sigma), applied 1/5000 in 3% BSA in PBS for 90 min at room temperature, followed by a fluorescent anti-mouse ALEXA 568 (Molecular Probes Inc., Eugene, OR, USA) antibody, applied 1/500 in 3% BSA in PBS for 90 min at room temperature. Confocal optical section stacks were collected using a Bio-Rad MRC-1000 confocal laser scanning microscope as described previously (Thompson *et al.*, 1997).

Specimen processing and electron microscopy

The sections on multi-well slides were dehydrated through an ethanol series and infiltrated in LR white resin (Agar Scientific, Stansted, Essex, UK), containing 0.5% benzoin methyl ether as catalyst, in a series 1 : 1, 1 : 2, 1 : 3 ethanol : resin and pure resin for 1 h each, and finally 100% resin overnight at -20°C. The slides containing the sections were taken out of the resin, and a gelatin capsule filled with resin containing the catalyst was inverted over each well. The resin was allowed to polymerize at room temperature overnight under UV light. The polymerized blocks were detached from the slide by cooling in liquid nitrogen.

Sections ranging from 100 nm to 1 µm were cut using a Leica Ultracut E and collected on 200 mesh grids coated with pyroxylin. Series of 0.5-µm-thick sections were collected on slot grids coated with formvar. The sections were stained with 2% (w/v) aqueous uranyl acetate for 40 min. Electron microscopy was carried out with a Jeol 1200 EX electron microscope running at 80 KV. The specimen holder was mounted on a eucentric goniometer stage that permitted grid tilting from -60° to +60° for stereo pair making.

3D Modelling of nucleolar DNA

Serial sections from pre-embedding labelled tissues were used for computer-assisted 3D modelling of nucleolar DNA. Micrographs of consecutive 0.5 µm sections across entire nucleoli were photocopied on transparencies for pairwise manual alignment on a light box. Then the original micrographs were trimmed down and scanned at 300 dpi (SCANMAKER 5, Microtek: <http://www.microtek.com>). 3D reconstructions were modelled using a Silicon Graphics computer running the IMOD software (Kremer *et al.*, 1996), available at <http://bio3d.colorado.edu>. On each section the outline of the nucleolus was contoured and each silver-gold particle was marked as a point. The nucleolar contours, the particles in the nucleolus and the extranucleolar labelling were considered as distinct objects, and a different colour (green, yellow and red, respectively) was assigned to each one.

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