

Whole mount fluorescence *in situ* hybridization (FISH) of repetitive DNA sequences on interphase nuclei of the small cruciferous plant *Arabidopsis thaliana*

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Hybridizing fluorescently labeled DNA probes *in situ* to the chromatin of interphase nuclei in whole mounts allows the study of nuclear architecture in morphologically well preserved specimens. Fluorescent labeling of the DNA probes (either directly or indirectly) allows the simultaneous, but differential, detection of several sequences (e.g. Lengauer et al., 1993; Nederlof et al., 1990). Also, fluorescent signals can be imaged by confocal microscopy so that stacks of optical section images can be recorded through the whole mounts. Multiple labeling FISH on whole mounts therefore allows the study of the relative positions of different chromosomes or chromosome segments in individual interphase nuclei as well as between nuclei of related cells.

In the experiments presented here, two tandemly repeated sequences, rDNA and a 500 bp repeat sequence, were hybridized to interphase nuclei of seedlings and flowers (inflorescences) of the small cruciferous plant *Arabidopsis thaliana*. The procedure used in the experiments, based on the protocols published by Ludevid et al. (1992) and Tautz and Pfeifle (1989), was described earlier by Bauwens et al. (1994).

I. Seed sterilization and germination

Note: Procedure I is based on the protocol of Valvekens et al. (1988).

Step	Action
1	Surface sterilize seeds of <i>Arabidopsis thaliana</i> (C24) by immersing them as follows: <ul style="list-style-type: none"> ▶ 2 min in 70% (v/v) ethanol. ▶ 15 min in a solution of 5% (v/v) NaOCl and 0.05% (v/v) Tween 20.
2	Wash seeds 5 times in sterile, distilled water.
3	Pipette onto germination medium (1x Murashige and Skoog salt mixture (Flow Laboratories, USA); 0.5 g/L 2-(<i>N</i> -morpholino)ethane sulphonic acid (MES), pH 5.7 (adjusted with 1 M KOH); 0.8% (w/v) Bacto-agar (Difco Laboratories, USA).
4	Allow the seeds to germinate at room conditions for 4 days.
5	Transfer part of the seedlings to soil.
6	Grow plants under continuous light conditions at desk temperature.
7	Harvest flowers and inflorescences after 3 – 4 weeks.

II. Tissue fixation

Step	Action
1	<p>Place approximately 30 – 40 seedlings or a few flowers or inflorescences in a glass vial containing:</p> <p>4.365 ml fixation buffer [1.1x PBS; 0.067 M EGTA, pH 7.5 (adjusted with NaOH)].</p> <p>Note: 10x PBS contains 1.3 M NaCl, 0.0027 M KCl, 0.07 M Na₂HPO₄, 0.03 M NaH₂PO₄; pH 7.2.</p> <ul style="list-style-type: none"> ▶ 0.135 ml 37% formaldehyde (Sigma, USA). ▶ 0.5 ml DMSO. <p>Note: Final concentrations in vial are 1% formaldehyde and 10% DMSO.</p>
2	Rock the glass vial for 25 min at room temperature.
3	<p>Remove the fixative and rinse as follows:</p> <ul style="list-style-type: none"> ▶ 2x with 5 ml methanol. ▶ 4x with 5 ml ethanol.
4	Discard the last ethanol wash, then cover sample with a final 5 ml ethanol.
5	<p>Leave sample at -20°C for 2 – 4 days.</p> <p>Caution: Keeping the material for longer periods of time in ethanol makes it brittle.</p>

III. Labeling of the probe DNA

Step	Action
1	<p>Use the following as hybridization probes:</p> <ul style="list-style-type: none"> ▶ A mixture of three ribosomal DNA (rDNA) inserts, each cloned into pBS (l)KS⁺ (Stratagene, USA) from <i>A. thaliana</i>. The inserts contain the 5.8S, 18S and 25S rRNA genes, as well as the intergenic region (IGR) (Unfried and Gruendler, 1990; Unfried et al., 1989). ▶ A 500 bp repeat DNA sequence, cloned into pGem-2 (Promega, USA). The repeat is one of three classes of highly repetitive, tandemly arranged DNA sequences in <i>A. thaliana</i> (Simoens et al., 1988).
2	<p>Label undigested samples of both probes according to the nick translation procedures in Chapter IV of this manual. Use the following labels:</p> <ul style="list-style-type: none"> ▶ Label the rDNA with either DIG-dUTP or fluorescein-dUTP. <p>Note: The concentration of substituted nucleotide in the nick translation labeling mixture should be the same for either fluorescein-dUTP or DIG-dUTP.</p> <ul style="list-style-type: none"> ▶ Label the 500 bp repeat DNA with DIG-dUTP.
3	<p>After nick translation, treat each labeled probe as follows:</p> <ul style="list-style-type: none"> ▶ Co-precipitate 1 µg of labeled DNA with 55 µg of sonicated salmon sperm DNA (Sigma, USA). ▶ Redissolve probe in 25 µl H₂O to a concentration of 40 ng labeled DNA per µl.

IV. Pretreatment

Step	Action
1	Remove ethanol from seedlings or flowers (or inflorescences) and transfer material to microcentrifuge tubes.
2	Fix each sample as follows: <ul style="list-style-type: none"> ▶ Rinse 2x with 1 ml ethanol. ▶ Replace ethanol with 1 ml ethanol/xylene (1:1) and incubate for 30 min. ▶ Rinse 2x with 1 ml ethanol. ▶ Rinse 2x with 1 ml methanol. ▶ Replace methanol with 1 ml of a 1:1 mixture of methanol and [PBT containing 1% (v/v) formaldehyde]. Rock for 5 min. <p>Note: PBT contains 1x PBS and 0.1% (v/v) Tween 20.</p>
3	Post-fix sample for 25 min in 1 ml PBT containing 1% formaldehyde.
4	Remove fixative and rinse sample with 5 x 1 ml PBT.
5	Wash sample 3 x 1 ml of 2x SSC (each wash, 5 min). Note: 1x SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0.
6	Digest with RNase A (100 µg/ml in 2x SSC) for 1 h at 37°C.
7	Wash 3 x 5 min with 1 ml PBT.
8	Digest with Proteinase K (40 µg/ml in PBT) for 8 min at 37°C.
9	After the Proteinase K digestion, do the following: <ul style="list-style-type: none"> ▶ Rinse 2x with 1 ml PBT. ▶ Wash 2 x 2 min with 1 ml PBT. ▶ Rinse 2x with 1 ml PBT.
10	Postfix a second time for 25 min with 1 ml PBT containing 1% formaldehyde.
11	Remove fixative and rinse 5x with 1 ml PBT.

V. *In situ* hybridization

Step	Action
1	Wash each sample 10 min with 1 ml of a 1:1 mixture of PBT and hybridization solution [hybridization solution contains 50% formamide (Ultra Pure from USB, USA) in 2x SSC].
2	Rinse sample with 2 x 1 ml hybridization solution.
3	<p>Remove the hybridization solution and add the following to each sample (to produce a final volume of 500 µl of hybridization solution):</p> <ul style="list-style-type: none"> ▶ 250 µl formamide. ▶ 50 µl 20x SSC. ▶ Enough H₂O to make a total volume, including probes, of 500 µl. ▶ 25 µl of each labeled rDNA probe (for single or double labeling experiments). ▶ 25 µl labeled 500 bp repeat probe (for double labeling experiments only). <p>Note: Each labeled probe has a final concentration of 2 ng/µl.</p> <p>Note: This incubation mixture can be used for either a single label hybridization (fluorescein-labeled probe) with direct detection; a single label hybridization (DIG-labeled probe) with indirect detection; or a double label hybridization (both fluorescein- and digoxigenin-labeled probes). See Procedure VIII below for details on these different types of experiments.</p>
4	<p>Treat the sample as follows:</p> <ul style="list-style-type: none"> ▶ Denature target and probe in hybridization solution for 4 min at 100°C. ▶ Place immediately on ice for 3 min. ▶ Centrifuge very briefly. ▶ Incubate overnight at 37°C to hybridize probe and target. <p>Caution: If using a directly labeled (i.e., fluorescent) probe, perform the hybridization incubation and the rest of the procedure in the dark.</p>

VI. Pre-absorption of antibodies (for indirect detection only)

Step	Action
1	<p>Prepare powdered <i>A. thaliana</i> root or seedling extract as follows:</p> <ul style="list-style-type: none"> ▶ Grind the root or seedling under liquid nitrogen. ▶ Extract the ground powder with acetone under liquid nitrogen. ▶ Decant the acetone supernatant. ▶ Let the residual acetone evaporate from the precipitate. ▶ Use the dry, powdered precipitate in the pre-absorption procedure below.
2	Dilute each detection antibody, in 4x SSC containing 1% (w/v) BSA, to the working dilution suggested by the manufacturers and a final volume of 500 µl.
3	Add approximately 2 mg of powdered <i>A. thaliana</i> root or seedling extract (from Step 1 above) to the diluted antibody.
4	Pre-absorb the antibodies overnight at 15°C, in the dark.
5	Centrifuge the pre-absorption mixture.
6	Use the supernatant in the immunocytochemical detection reaction.

VII. Posthybridization washes

Step	Action
1	<p>After overnight hybridization at 37°C (Procedure V, Step 4), treat the hybridization sample as follows:</p> <ul style="list-style-type: none"> ▶ Remove the hybridization solution. ▶ Wash the sample for 1 h in fresh hybridization solution at 37°C. ▶ Wash the sample 4 x 30 min in hybridization solution at 37°C. ▶ If performing an <i>in situ</i> hybridization experiment with directly labeled probe DNA (rDNA) in a single labeling experiment, proceed to procedure VIII a. ▶ If performing an <i>in situ</i> hybridization experiment requiring indirect detection with antibodies, proceed to step 2.
2	<p>Bring the seedlings or flowers (or inflorescences) gradually to 4x SSC through the following washes (all at room temperature):</p> <ul style="list-style-type: none"> ▶ 20 min with a 3:1 mix of hybridization solution and 4x SSC. ▶ 20 min with a 1:1 mix of hybridization solution and 4x SSC. ▶ 20 min with a 1:3 mix of hybridization solution and 4x SSC. ▶ 4 x 5 min with 4x SSC.

VIII. Immunocytochemical detection

Use the immunocytochemical detection schemes detailed in Table 1 to analyze the single and double labeling *in situ* hybridization experiments.

Type of Experiment	Probe	Label	Antibody ^a		Procedure to Follow
			1st (detecting)	2nd (amplifying)	
Single label, direct detection	rDNA	Fluorescein-dUTP	–	–	VIIIa
Single label, indirect detection	rDNA	DIG-dUTP	Fluorescein-conjugated anti-DIG antibody (from sheep) ^b	Fluorescein-conjugated anti-sheep antibody (from donkey) ^c	VIIIb
Double label	rDNA 500 bp	Fluorescein-dUTP DIG-dUTP	– Tetramethylrhodamine-conjugated anti-DIG antibody from sheep) ^d	– Tetramethylrhodamine-conjugated anti-sheep antibody body (from rabbit) ^e	VIIIb

Table 1: Immunocytochemical detection schemes for single and double labeling experiments

^a All antibodies should be pre-absorbed, according to Procedure VI above.

^b Fab fragments.

^c Fab fragments, from Sigma, USA.

^d Fab fragments.

^e Whole molecule, from Chemicon, USA.

VIIIa. Direct detection

Step	Action
1	Bring the samples to 1x PBS gradually through the following washes (all at room temperature): <ul style="list-style-type: none"> ▶ 20 min with a 3:1 mix of hybridization solution and 1x PBS. ▶ 20 min with a 1:1 mix of hybridization solution and 1x PBS. ▶ 20 min with a 1:3 mix of hybridization solution and 1x PBS. ▶ 4 x 15 min in 1 ml 1x PBS.
2	Proceed to the staining and mounting procedure (Procedure IX).

VIIIb. Indirect detection

Step	Action
1	After the posthybridization washes (Procedure VII), remove the 4x SSC solution from the samples.
2	Add the first pre-absorbed antibody (from Table 1) to the sample and incubate overnight at 15°C in the dark.
3	Remove the first antibody and wash the material 4 x 15 min in 1 ml 4x SSC containing 0.05% (v/v) Tween 20.
4	Depending on whether you are using an amplifying (second antibody), do either of the following: <ul style="list-style-type: none"> ▶ If you use an amplifying antibody, proceed to Step 5. ▶ If you do not use an amplifying antibody, proceed to Step 7.
5	If amplifying the signal, wash the sample 4 x 15 min with 4x SSC containing 1% bovine serum albumin.
6	Remove the wash solution; add the second pre-absorbed antibody (from Table 1) to the sample, and incubate overnight at 15°C in the dark.
7	After the last (first or second) antibody incubation, remove the antibody and wash the sample 4 x 15 min in 1 ml 1x PBS.
8	Proceed to the staining and mounting procedure (Procedure IX).

IX. Staining and mounting

Step	Action
1	Counterstain the sample as follows: <ul style="list-style-type: none"> ▶ For single labeling experiments with fluorescein-conjugated antibodies or nucleotides: Counterstain the chromatin of the interphase nuclei with 0.5 µg/ml propidium iodide (in 1x PBS) for 1 h in the dark. ▶ For double labeling experiments with fluorescein- and tetramethylrhodamine-conjugated nucleotides and antibodies: Counterstain the chromatin of the interphase nuclei with 0.2 µg/ml DAPI (in 1x PBS) for 1 h in the dark. <p>Note: DAPI is 4,6'-diamidino-2-phenylindole.</p>
2	Place a few seedlings or flowers (or part of an inflorescence) on a slide.
3	Apply some tape to the slides to create a support for the cover slip, so as not to crush the seedlings or flowers.
4	Apply a drop of antifade reagent (Vectashield, Vector Laboratories, USA) and cover with a cover slip.

X. Fluorescence microscopy

Xa. Conventional fluorescence microscopy

Step	Action
1	Visually inspect the slides on a DIAPHOT 300 inverted microscope (Nikon, Japan), fitted with either a 60x, NA 1.40 oil immersion lens (Olympus, Japan) or an NPL FLUOTAR, 40x, NA 1.30 oil immersion lens (Leitz, FRG).
2	Use the following filter combinations (Chroma Technology Corp., USA): <ul style="list-style-type: none"> ▶ To localize propidium iodide-stained nuclei and tetramethylrhodamine-labeled hybrids: filter block 31014 404. ▶ To localize DAPI-stained nuclei: filter block 31000 404. ▶ To localize fluorescein-labeled hybrids: filter block 31001404.
3	As light source, use a mercury arc lamp (100 W).

Xb. Confocal fluorescence microscopy

Step	Action
1	Record images with the MRC-600 Confocal Scanning Laser Microscope (CSLM) System (Bio-Rad, USA). Attach the CSLM to the DIAPHOT 300 inverted microscope fitted with appropriate lenses (same microscope and lenses as described in Procedure Xa, Step 1 above).
2	Use the K1 / K2 filter block combinations (Bio-Rad, USA) and either: <ul style="list-style-type: none"> ▶ The 568 nm line from a Krypton-Argon laser (Ion Laser Technology, Utah, USA), to image the propidium iodide-stained interphase nuclei and the tetramethylrhodamine-labeled hybrids. ▶ The 488 nm line from the same Krypton-Argon laser, to image the fluorescein-labeled hybrids.

Results and discussion

Figures 1 and 2 show some of the results obtained by FISH of rDNA and the 500 bp repeat on whole mounts of flowers and seedlings from *A. thaliana*.

The rDNA from *A. thaliana* covers about 5.7 Mbp per haploid genome (Meyerowitz and Pruitt, 1985), and is distributed over two large tandem repeats on chromosomes 2 and 4 (Maluszynska and Heslop-Harrison, 1991; Murata et al. 1990). Diploid interphase nuclei from *A. thaliana* exhibit, however, a number of rDNA-loci ranging from two to more than four (Bauwens et al. 1991) as can clearly be seen from the merged image in Figure 1.

The 500 bp repeat sequence covers about 0.3 – 0.6 Mbp per haploid genome (Bauwens et al., 1991; Simoens et al., 1988) and exhibits a chromosome-specific large cluster (Bauwens and Van Oostveldt, 1991; Bauwens et al., 1991), resulting in two distinct signals in diploid interphase nuclei as can be seen from the merged image in Figure 2.

Some helpful remarks should be made about confocal observation of fluorescent signals in whole mounts.

Sequential excitation with the 568 nm and the 488 nm lines of the Kr-Ar-laser and the K1/K2 filter combinations from the Bio-Rad MRC-600 CSLM, allowed a clear separation of the red (tetramethylrhodamine) and the green (fluorescein) signal. [See the signals from the 500 bp repeat (red) and the rDNA (green) probes in Figure 2.]. Especially, the yellow 568 nm line allows specific excitation of the red fluorescing dyes such as tetramethylrhodamine or, preferably, Texas Red with almost no excitation of fluorescein.

Confocal observation of the preparations appeared to be absolutely necessary to obtain clear images of the signals, especially in the very dense meristematic tissues of the seedling roottips and the developing flowers in the inflorescences. This was especially true for the weaker signal of the 500 bp repeat that, in many cases, could not be observed through the autofluorescence haze by conventional fluorescence microscopy. Acquiring digital images through confocal microscopy has the added advantage that images recorded at different wavelengths can be easily and accurately merged and compared.

Finally, the development of FISH protocols on whole mounts for localizing mRNA sequences, as already suggested by de Almeida Engler et al. (1994), will make it possible to follow the expression of different genes simultaneously at the cellular level, in three dimensions, through confocal observation.

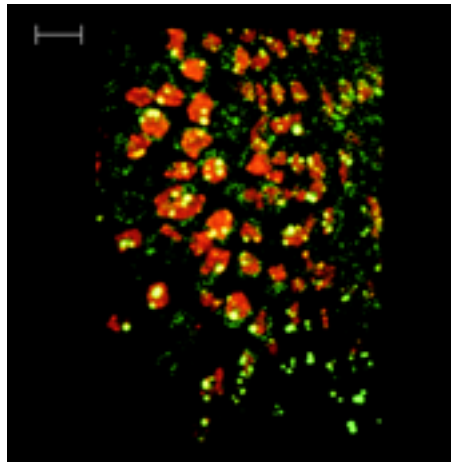


Figure 1: Whole mount FISH with rDNA on a developing flower from *A. thaliana*. The image is a merged image representing part of the pistil of a developing flower, showing fluorescein-labeled rDNA loci (yellow-green) and propidium iodide-stained interphase nuclei (red). An extended focus image of 15 optical sections through the pistil was created by maximum brightness projection before merging the 'green' (rDNA) and 'red' (nuclei) images. Bar 25 μm \triangleq 17 mm.

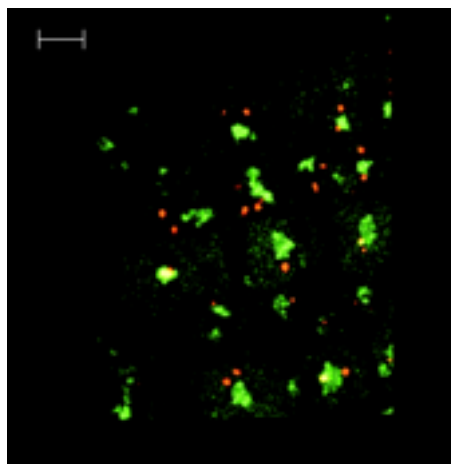


Figure 2: Whole mount double labeling FISH with rDNA and the 500 bp repeat on a seedling from *A. thaliana*. This merged image represents part of the meristematic zone of a seedling roottip. It shows fluorescein-labeled rDNA loci (green) and tetramethylrhodamine-labeled 500 bp repeat loci (red). An extended focus image of 20 optical sections through the meristematic zone of the roottip was created by maximum brightness projection before merging the 'green' (rDNA) and 'red' (500 bp repeat) images. Bar 25 μm \triangleq 15 mm.

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Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
DIG-Nick Translation Mix	For generation of highly sensitive probes for <i>in situ</i> hybridization with digoxigenin-11-dUTP. Premixed solution for 40 labeling reactions	1 745 816	160 µl
Nick Translation Mix	For generation of highly sensitive probes for fluorescence <i>in situ</i> hybridization. The Nick Translation Mix for <i>in situ</i> probes is designed for direct fluorophore-labeling of <i>in situ</i> probes.	1 745 808	200 µl
Fluorescein-12-dUTP	Tetralithium salt, solution, 1 mmol/l	1 373 242	25 nmol (25 µl)
dNTP Set	Set of dATP, dCTP, dGTP, dTTP, lithium salts, solutions	1 277 049	1 set, 4 x 10 µmol (100 µl)
Anti-Digoxigenin-Rhodamine	Fab Fragments from sheep	1 207 750	200 µg
Anti-Digoxigenin-Fluorescein	Fab Fragments from sheep	1 207 741	200 µg
Tween 20		1 332 465	5 x 10 ml
Proteinase K	Lyophilizate	161 519 745 723 1 000 144 1 092 766	25 mg 100 mg 500 mg 1 g
RNase A	Dry powder	109 142 109 169	25 mg 100 mg