

GENERATION OF ARABIDOPSIS LEAF NUCLEI FOR PROTEIN IMMUNOLOCALIZATION OR DNA FISH

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REPRESENTATIVE PUBLICATIONS:

Pontes, Olga, Carey Fei Li, Pedro Costa Nunes, Jeremy Haag, Thomas Ream, Alexa Vitins, Steven E. Jacobsen and Craig S. Pikaard (2006). The Arabidopsis chromatin-modifying nuclear siRNA pathway involves a nucleolar RNA processing center. *Cell* 126: 79-92.

Richard J. Lawrence, Keith Earley, Olga Pontes, Manuela Silva, Z. Jeffrey Chen, Nuno Neves, Wanda Viegas and Craig S. Pikaard (2004). A concerted DNA methylation/histone methylation switch regulates rRNA gene dosage control and nucleolar dominance. *Molecular Cell* 13: 599-609.

Pontes, Olga, Richard J. Lawrence, Nuno Neves, Manuela Silva, Jae-Hyeok Lee, Z. Jeffrey Chen, Wanda Viegas, and Craig S. Pikaard (2003). Natural variation in nucleolar dominance reveals the relationship between nucleolus organizer chromatin topology and rRNA gene transcription in Arabidopsis. *Proc. Natl. Acad. Sci. USA* 100:11418-11423.

Pontes, Olga, Nuno Neves, Manuela Silva, Michelle S. Lewis, Andreas Madlung, Luca Comai, Wanda Viegas and Craig S. Pikaard (2004). Chromosomal locus rearrangements are a rapid response to formation of the allotetraploid Arabidopsis suecica genome. *Proc. Natl. Acad. Sci. USA* 101: 18240-18245.

Materials and Reagents

-Nuclear extraction buffer (NEB)

10 mM Tris-HCl pH 9.5

10 mM KCl

0.5 M sucrose

4 mM spermidine

10 mM spermine

0.1% 2-mercaptoethanol

- 8% formaldehyde (or paraformaldehyde). Formaldehyde comes as a 37% stock (Sigma), so one dilutes ~4.5 fold to make 8% formaldehyde.

- Nylon mesh with mesh sizes of 20 (optional), 50- and 100 μm .

-Plastic petri dishes

-razor blades

-Glass slides (we use Fisherbrand frosted slides) and coverslips

- DAPI (4'-6-Diamidino-2-phenylindole) in CITIFLUOR antifade solution (1 $\mu\text{g}/\mu\text{l}$)

purchased from Electron Microscopy Sciences, Hatfield, PA

Preparation of nuclei

- In the lid of a plastic petri dish, lying on a bed of ice, Chop 1g of fresh leaf tissue from ~3 week old plants grown in soil. Rosettes of these plants are ~ 3 cm in diameter. Choose intermediate-sized leaves (neither the oldest nor youngest) for protein immunolocalization, but choose smaller, newly emerged leaves for RNA FISH detection of siRNAs). Chop the leaves in 1-2 ml of NEB using a razor blade until you get as fine a suspension as possible. This will take 5 minutes or more of vigorous mincing. Keep in mind that you are trying to chop the tissue sufficiently that nuclei are released from disrupted cells, so the tissue must be chopped extremely fine.

-Using a blue pipette tip, with its tip cut off to make the bore wider, pipette the homogenate from the lid of the petri dish into a 15 ml conical tube, on ice.

- add a volume of 8% formaldehyde that is equal to the volume of NEB used for homogenation. Mix by swirling and allow nuclei/tissue to fix for 20 min, on ice.

-Meanwhile, make filters from blue pipette tips and nylon mesh of 50 and 100 μ m:

 Cut ~3 cm off the tips of blue pipette tips, where the bore is not yet full width, such that the cut tip can fit most of the way into a 1.5 ml microcentrifuge tube. Place the cut tip near a blue flame, while twirling, for 1-2 seconds to melt the plastic slightly and while still molten, push the tip down onto a sheet of 50 or 100 μ m nylon mesh so that the mesh adheres to the tip. Trim off excess mesh material. The filter tip will now just fit into the top of a 1.5 ml microcentrifuge tube. Make both 50 and 100 μ m filters by this method.

- Filter the homogenate first through the 100 μ m filter and then through the 50 μ m filter.

- Centrifuge the homogenate 3 min at only 2.5rpm at 4°C. You should see a whitish/grayish smudge (the nuclei!) at the center of a green pellet

- Resuspend the pellet in 40 μ l of NEB. The white/gray smudge will be the hardest part to resuspend.

- Apply 3 μ l onto individual slides, near the end of the slide furthest from the frosted writing surface. Don't go over the same area twice, but go back and forth in adjacent parallel passes to achieve a thin film covering a 2-3 cm square. Slides will dry quickly in the air.

- Check the quality of the nuclei by adding a drop of DAPI (in CITIFLUOR). Cover with a coverslip and store in the dark for several hours. Cardboard slide storage books are useful for this step.

- Slides can be stored at 4°C for up to one month.

IMMUNOSTAINING OF CHROMOSOMAL PROTEINS IN PLANT CELLS

Solutions and reagents

- 4% PFA (paraformaldehyde) or formaldehyde
- 10X KPBS
 - 1.28M NaCl
 - 20mM KCl
 - 80mM Na₂HPO₄
 - 20mM KH₂PO₄ (pH 7.2)
- DAPI at 1µg/µl in CITIFLUOR
- Triton X-100
- Blocking Solution: 1% BSA Fraction V in KPBS 0.1% Triton X
- Primary and secondary antibodies, typically 1:200 to 1:1000 dilutions in Blocking Solution.

Primary antibody incubation

- (Optional) re-fix preparations in formaldehyde 30 min and wash 3X 5min in KPBS/0.1% Triton X.
- Block preparation by placing 200 µl blocking solution onto the slide and cover with a 22x22 mm coverslip. Incubate in a humid chamber for 30 min at 37°C.
- Wash off blocking solution 3X 5min in KPBS/0.1% Triton X.
- Add primary antibody solution (50-100 µl per slide) in blocking solution. Cover with a 22x22 mm coverslip and place in the humid chamber overnight at 4°C.

Detection, Counterstaining and Mounting

- Wash 3X 5 min in KPBS.
- Block with blocking solution 30 min, in a humid chamber for 30 min at 37°C.
- Add the proper secondary antibody diluted in KPBS/0.1% Triton X, cover with 22x22 mm coverslip and incubate 2h at 37°C.
- Wash 3X 10 min in KPBS and mount the slides in DAPI and cover with a coverslip 22x40 mm. Keep at 4°C.

FLUORESCENT IN SITU HIBRIDIZATION (FISH)

Solutions and reagents

- 10 mM HCL
- Pepsin
10 mg/ ml in 10 mM HCL
- RNase A
100µg/ml in 10 mM Tris.HCl, pH 8.0)
- Tween20
- 20X SSC
3M NaCl
0.3M Sodium Citrate (pH 7.5).
- 4X SSC/0.2% Tween-20
- 2X SSC
- Formamide
- Dextran sulfate
- SDS (sodium dodecyl sulfate)
- Salmon sperm
- Blocking solution: 1% BSA Fraction V in 4X SSC/0.2% Tween20
- Detection solution: Digoxigenin (Fab fragments) conjugated to FITC (dilution 1:50 of 200 µg/ml) for digoxigenin labeled probes; and Cy3-Streptavin (1:500 of 1 mg/ml) in the blocking solution.
- DAPI at 1µg/µl in CITIFLUOR antifade solution

DNA probe labeling

Nick Translation (Roche Kit), according to the manufacturer's description, or by PCR.

Pretreatment of chromosome preparations

- Incubate the preparations for 10 min at 37°C, in a humid chamber, with 200 µl pepsin for each slide. Cover with a cover slip (22x22 mm), and wash 3X in 2xSSC.
- Add 200µl of RNase A to each slide. Cover with a cover slip (22x22 mm), incubate in a humid chamber for 1h at 37°C, and then wash 3X in 2XSSC.
- Post-fix 10 min at room temperature in 4% formaldehyde. After this treatment wash the slides 3X in 2X SSC.
- Dehydrate slides through a 70%, 90% and 100% ethanol series, 3 min each, and air-dry.

In situ hybridization mixture and hybridization conditions

The hybridization mixture is prepared according to optimized conditions, found empirically. The stringency at which an *in situ* experiment is carried out reflects the approximate percentage of nucleotides that are correctly matched in the probe and the target. Stringency depends on the GC content, length of the probe, salt concentration, formamide concentration and the reaction temperature. The hybridization mixture contains sonicated salmon sperm, dextran sulfate and SDS (sodium dodecyl sulfate). A typical hybridization mixture is exemplified in the table I.

Table I. *In situ* hybridization mixture. Components and concentrations

Stock solution	Final concentration in mixture
100% formamide	50%
20X SSC	2X
50% dextran sulfate (w/v)	10%
10% SDS (w/v)	0.1%
Salmon sperm (10 µg/ml)	1.5 µg/µl
Probe	100-250 ng each
Sterilize water	Adjusted to a final volume of 20-30µl

- Add the hybridization mixture and cover with a coverslip 22x22 mm coverslip. Denature at 80°C for 5 min on a modified thermocycler (thermocycler JMRResearch PCT-100). Place the slides overnight in a humid chamber at 37°C; make sure the chamber is pre-warmed.

Post-hybridization washes and detection of hybridization sites

- Post hybridization washes are carried out using 20 % (v/v) formamide in 0.1X SSC at 42°C for 10 min.

-Wash the slides 2x 5min in 2X SSC at 42°C, and 2x in 4X SSC/0.2% Tween-20 at room temperature.

- Block the slides for 15 min at room temperature with blocking solution.

- The detection solution contains anti-digoxigenin (Fab fragments) conjugated to FITC or Cy3-Streptavin and the incubation is performed in humid chamber for 1h at 37°C.

- Wash the slides in 4x SSC/0.2% Tween-20 for 3x 5 min.

- Mount the slides in a drop of DAPI and cover with coverslip 22x40 mm. Keep at 4°C.