Fiber-FISH on nuclear DNA

Isolation of plant nuclei:

Isolation of plant nuclei is the same as for the preparation of high molecular weight DNA. The nuclei are suspended in 50% glycerol and stored at -20°C. Many published protocols call for a 20 or 30mm filtrations, but this can be omitted it with most plant materials in order to obtain the maximum number of nuclei. If there is too much debris in your suspension or on your slides the 20 mm filtration can be added.

1. Freeze 2-5 grams fresh leaf material in liquid nitrogen and grind to a fine powder with a pre-cooled (-20°C) mortar and pestle.
2. Transfer powder to a 50 ml centrifuge tube, add 20 ml chilled Nuclei Isolation Buffer (NIB) and mix gently (make sure to break up clumps) on ice for 5 min. [NIB: 10mM Tris-HCl pH9.5, 10mM EDTA, 100mM KCl, 0.5M sucrose, 4.0mM, spermidine, 1.0mM spermine, 0.1% mercapto-ethanol. Prepare a stock and store at 4°C. Mercapto-ethanol should not be included in the stock. It should be added just before use.].
3. Filter through nylon mesh: 220, 148,48 and 22 µm sequentially, into cold (on ice) 50 ml centrifuge tubes using a chilled funnel (Nylon filters obtained from: Tetko Inc, P.O. Box 346, Lancaster, NY, 14086, tele 914-941-7767.
4. Add 1ml NIB containing 10% (V/V) Triton X-100 (pre-mixed) and gently mix the filtrate. Final concentration of Triton X-100 should be 0.5%; itremoves any choloroplast contamination. Centrifuge at 2000 x g for 10 min at 4˚ C then decant the supernatant. If your pellet is very small, you may wish to skip the further cleaning steps and move directly to step 7. Otherwise, if you have a large pellet, you should resuspend the pellet in 20 ml NIB (with mercaptoethanol added).
5. Filter through nylon mesh: 48 and 30µm (optional) sequentially, into cold (on ice) 50 ml centrifuge tubes using chilled funnel. [You may perform a the 22µm filter step if you wish, but you will lose a lot of nuclei. However, this makes the nulei cleaner.]
6. Add 1ml NIB containing 10% (V/V) Triton X-100 (pre-mixed) and gently mix the filtrate, as in step 4. Centrifuge at 2000 x g for 10 min at 4˚ C.
7. Decant the supernatant and resuspend the pellet in 200 µl to 5 ml of 1:1 NIB:100% glycerol (neither mercaptoethanol nor Triton X-100 added) depending on the amount of nuclei harvested (Concentration ~5x106 nuclei/ ml, can be checked by staining with DAPI and examining under a microscope). Store at -20˚ C.

Extension of DNA fibers:

Extending the fibers is a critical step in Fiber-FISH for which there are several published methods. We have chosen the method of dragging with a coverslip as it seems to give the most uniform results. When dragging it is imperative that it be done slowly and smoothly. We also use poly-L-lysine slides obtained from Sigma. These slides are treated so as to promote the adhesion of the DNA molecule. Silinated slides can also be used but seem to generate more background signal. The calibration of the method should be checked occasionaly by using BACs or cosmids of a known length as probes.

1. Identify the nuclei portion in the nuclei stock. The nuclei tend to settle near the very bottom of the tube and in my experience the settling process can take a day or longer. The very bottom of the tube may appear very white and the nuclei oftentimes sit right above this bottom film. The color of the nuclei is variable across species and samples, but very clean nuclei is normally a gray/white coloration. Layers above the nuclei tend to contain debris and anything floating above in the storage solution is debris. Some people like to mix the nuclei stock prior to slide preparation by gently inverting the eppendorf tube several times. This is normally avoided as it mixes the debris with the nuclei.
2. With a cut P20 pipet tip, pipet 1-10µl nuclei suspension (1-5 µl/slide depending on the suspension concentration) into ~100µl NIB (minus mercaptoethanol and Triton) in an eppendorf tube to dilute the glycerol. Gently mix the nuclei with the buffer and centrifuge at 3000-3600 rpm for 5 min. Carefully remove the supernatant with a pipet leaving the nuclei pellet.
3. Resuspend the nuclei in PBS such that the final volume is 2 µl per slide. [PBS: 10mM sodium phosphate, pH7.0, 140mM NaCl]
4. Pipet 2 µl suspension across one end of a clean poly-L-lysine slide (Sigma, Poly-Prep, Cat # P0425) and air dry for 5-10 minutes. The nuclei should dry to the point where it appears "sticky", niether wet nor overdried.
5. Pipet 8 µl STE lysis buffer on top of the nuclei and incubate at room temperature for 4 min. [STE: 0.5% SDS, 5mM EDTA, 100mM Tris, pH7.0]
6. Slowly drag the solution down the slide with the edge of a clean coverslip held just above the surface of the slide; do not touch the coverslip to the slide surface as this will drag the nuclei completely off the slide. Air dry for 10 min.
7. Fix in fresh 3:1 100% ETOH: glacial Acetic Acid for 2 min.
8. Bake at 60˚C for 30 min.
9. Slides are best when used immediately but can be stored for several weeks.

Probe application:
Probe is prepared as described in Jiang (1996) and 10ml is applied to the slide, covered with a 22x22 mm coverslip and sealed with rubber cement. After the cement dries the slide is placed in an 80°C oven for 3 min in direct contact with a heated surface, then for 2 min in a wet chamber pre-warmed in the 80°C oven. The wet chamber, with the slides, is immediately transferred to 37°C chamber overnight. For difficult probes, the hybridization time can be several days in the 37°C wetchamber.

Probe detection:
Three layer detection gives much stronger signal than does a single layer of antibodies. All antibody layers are composed of the antibodies diluted in the appropriate buffers at the concentration specified below. 100µl are applied to each slide and a 22x40 cover slip is gently placed upon the antibody solution to promote even spreading. All antibody layers are incubated in a 37°C wet-chamber for a minimum of 30 min. Often the first layers are incubated for up to 45-60 minutes. Additional notes: The blocking step using 4M buffer seems to help reduce some of the background noise, but may be omitted at the users discretion. Dry bovine milk

from Sigma works the best in the 4M buffer, other substitutes (i.e. Carnation dry milk) also help to reduce the amount of signal. The 4M and TNB buffers can be prepared at 5X and stored at -20°C. The wash solutions, 4T and TNT, can be prepared at 20X and 10X, respectively, and stored at room temperature.

One-Color Detection Protocol

		Time (min)
1.	wash in 2X SSC	5
	wash in 2X SSC 42˚ C	10
	wash in 2X SSC	5
2.	wash in 1X 4T	5
	incubate at 37˚C in 4M	30
	wash in 1X 4T	2
3.	Incubate FITC-Avidin (/100µl TNB buffer)	30
	wash 3x in 1X TNT	5 (15)
4.	Incubate Biotin anti-avidin (100µl TNB buffer)	30
	wash 3x in 1X TNT	5 (15)
5.	Incubate FITC-Avidin (100µl TNB buffer)	30
	wash 3x in 1X TNT	5 (15)
	wash 2x in 1X PBS	5 (10)

6. Add 10ml Prolong (Molecular Probes) or Vectashield (Vector Labs), cover with a 22x30 mm coverslip and squash.

Solutions:

4M: 3 to 5% nonfat dry milk [Sigma, Cat # M7409] in 4 x SSC

4T: 4 x SSC, 0.05% Tween 20

TNB: 0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.5% blocking reagent (Boehringer Mannheim)

TNT: 0.1M Tris-HCl, 0.15M NaCl, 0.05% Tween 20, ph7.5

PBS: 0.13M NaCl, 0.007M Na2HPO4, 0.003M NaH2PO4

Two-Color Detection Protocol

		Time (min)
1.	wash in 2X SSC	5
	wash in 2X SSC 42˚ C	10
	wash in 2X SSC	5
2.	wash in 1X 4T	5
	incubate at 37˚C in 4M	30
	wash in 1X 4T	2
3.	Incubate FITC anti-dig (100µl TNB buffer) + Texas Red streptavidin (100µl), 37˚C for 30m	
	wash 3x in 1X TNT	5 (15)
4.	Incubate FITC anti-sheep (100µl TNB buffer) + Biotinylated anti-avidin (100µl), 37˚C for 30 min.	
	wash 3x in 1X TNT	5 (15)
5.	Incubate Texas Red streptavidin (100µl TNB buffer), 37˚C for 30 min.	
	wash 3x in 1X TNT	5 (15)

wash 2x in 1X PBS 5 (15)

6. Add 10ml Vectashield (Vector Labs), cover with a 22x30 mm coverslip and squash.

Poly-L-lysine slide preparation

1. Boil slides in 5 M HCl for 2 to 3 hrs.
2. Rinse thoroughly with dH2O then air dry
3. Incubate overnight in filtered 10-6 g/ml poly-D-lysine (MW=350,000, Sigma)
4. Rinse thoroughly

Slide silanation

1. 30 min. 1:1 Hcl:methanol
2. overnight 18M Sulfuric Acid
3. 8-10 washes in ddH20
4. 10 min. boiling ddH20
5. 1 hr. 10% 3-aminopropyltriethoxy silane in 95% ETOH
6. rinse several times in ddH20
7. wash in 100% ETOH
8. 80-100°C overnight before use

BAC Fiber-FISH

Prior to preparing slides:

1 Label appropriate probes with biotin and dig (see Probe Labelling protocol).

2. Mini-prep BAC DNA (Alkaline lysis method; use 20 µl water for resuspension).

3. Silanize 22x22 coverslips by dipping in Sigmacote for 10 min, then air dry.

Slide preparation:

4. Prepare wet-chamber at 37°C, turn slide warmer up to 60°C.

5. Dilute BAC DNA (w/ cut P20 pipet tips) to appropriate level (We like to dilute 1 µl BAC into 9 µl water). Add all 10 µl of diluted BAC to Poly-Prep slide (Sigma #P0425).

6. Add 15 µl of FISH lysis buffer* to BAC drop. Allow drop to spread. Let this sit at room temperature for ~5 min. Add water to the slide if it dries.

7. Gently place ("drop") a silanized coverslip directly over the liquid (lower the slide with forcepts to avoid bubbles).

8. Transfer slides to slide warmer. Allow slides to "bake" for 15 min. At this point one should see the liquid begin to recede.

9. Place slides in 3:1 (EtOH: Glacial Acetic Acid), wait 1 min. Gently shake slide to promote removal of the coverslip. Once coverslip falls off, transfer slides to new container of 3:1 and incubate for 1:30. Transfer slides back to slide warmer for an additional 15 min.

10. Add probe, denature, and detect as in Nuclear Fiber-FISH protocol.

*FISH lysis buffer :

2% Sarkosyl
0.25% SDS
50 mM Tris (pH 7.4)

50 mM EDTA (pH 8.0)