

## Fluorescence in Situ Hybridization Techniques for Cytogenetic and Genomic Analyses

Jason G. Walling, Wenli Zhang, and Jiming Jiang

### Abstract

Fluorescent in situ hybridization (FISH) is a powerful method to visualize DNA sequences in the context of the whole chromosome. Yet despite the value of FISH analysis for cytogenetic studies, there are surprisingly few labs that are able to adapt the technique for their experiments in chromosomal and genome biology. Here we present a comprehensive FISH protocol acquired from over 20 years of collective experience using different plant species. Our description uses rice as a model for performing a complete FISH procedure, but the protocol can be readily adapted for other plant species. We have provided more specialized instruction beyond routine FISH, which includes the preparation of meiotic and mitotic samples suitable for FISH analysis, procedures for direct and indirect labeling of DNA probes, and techniques for increasing signal strength using layers of antibodies.

**Key words:** Fluorescent in situ hybridization, Mitotic metaphase chromosomes, Pachytene chromosomes

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### 1. Introduction

Currently there are two popular methodologies for developing physical maps in plant genome research. The first is to develop a bacterial artificial chromosome (BAC) library in which all clones are fingerprinted and contiged (1). This approach generates a high-resolution genome-wide physical map. However, it also requires genetic and genomic resources to anchor BAC contigs to individual chromosomes. The second methodology is to map DNA clones, such as BACs, to individual chromosomes by fluorescence in situ hybridization (FISH) (2, 3). FISH essentially is a manual technique with inherently low throughput. FISH-based physical maps have a limited and sometimes variable resolution depending on the cytological targets (somatic metaphase chromosomes or meiotic pachytene chromosomes). However, FISH as a physical mapping

tool has several beneficial advantages that cannot be replaced by other techniques. First, DNA sequences can be directly mapped to specific chromosomes. Second, two distinct types of chromatin, euchromatin and heterochromatin, can be differentially visualized at mitotic interphase and/or at the meiotic pachytene stage. Thus, FISH can anchor DNA sequences to a specific euchromatin/heterochromatin domain.

Rice has been established as one of the best model species for cytogenetic research. The 12 rice pachytene chromosomes are well differentiated from each other (4–7) and provide excellent cytological targets for FISH mapping (3, 8). The rice genome is relatively small and contains a limited amount of repetitive DNA sequences compared to other cereal crops. Therefore, most large-insert genomic DNA clones, such as BACs, can be readily used as FISH and/or fiber-FISH probes (2, 9). In contrast, a significant proportion of genomic clones from species with larger and more complex genomes, such as maize and wheat, often cannot be used as FISH probes because the repeats within the clones generate cross-hybridization signals that mask the true locations of the FISH probes.

FISH as a tool for cytogenetic and genomic research has been extensively used in both rice cytogenetic and genomic research. Cytological targets used in rice FISH mapping include interphase nuclei (2, 10), somatic metaphase chromosomes (2, 11, 12), pachytene chromosomes (3, 7, 8), and DNA fibers (13, 14). In this chapter we provide a comprehensive FISH protocol that can be applied to all of these cytological targets. Procedures including probe labeling, FISH, FISH detection, and signal amplification using layered antibodies are outlined. Furthermore we provide in-house technical notes that are key to the success of FISH experiments. Such technical notes are usually not provided in standard research papers.

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## 2. Materials

### 2.1. Chromosome Preparation

1. Rice Seeds (*Oryza sativa* ssp. *japonica* cv. Nipponbare).
2. 2 mM 8-hydroxyquinoline: 0.15 g 8-hydroxyquinoline, 500 ml ddH<sub>2</sub>O. Store in the dark at 4°C.
3. Fixation Solution: 3 parts 100% EtOH, 1 part glacial acetic acid. Make fresh.
4. 45% Acetic Acid.
5. Digestion Cocktail: 1% (w/v) Pectinase from *Aspergillus niger* (Fluka), 2% (w/v) Cellulase “Onozuka” R-10 (Yakult), in 1× PBS. Store at 4°C.
6. Microscope Slides (Fisherbrand Premium).
7. 18×18 or 22×22 mm glass coverslips (Fisherbrand).

8. Fine Forceps.
9. Alcohol burner.
10. Acetocarmine.
11. Phase contrast microscope.

## **2.2. Probe Labeling**

1. Labeled dNTP solution: 0.166 mM dUTP (conjugated with biotin, dig, or fluorophore), 0.333 mM dTTP. Store at  $-20^{\circ}\text{C}$ .
2. dNTP solution: 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP. Store at  $-20^{\circ}\text{C}$ .
3. 10 $\times$  Nick Translation buffer: 0.5 M Tris-HCl pH 7.5, 50 mM  $\text{MgCl}_2$ . Store at  $-20^{\circ}\text{C}$ .
4. DNase I stock buffer: 10 mM HEPES, pH 7.5, 50% (V/V) glycerol, 10 mM  $\text{CaCl}_2$ , 10 mM  $\text{MgCl}_2$ . Store at  $-20^{\circ}\text{C}$ .
5. DNase I dilution buffer: 10 mM HEPES, pH 7.5, 15% (V/V) glycerol, 10 mM  $\text{CaCl}_2$ , 10 mM  $\text{MgCl}_2$ . Store at  $-20^{\circ}\text{C}$ .
6. DNase I.
7. DNA Polymerase I.
8. Plasmid or Genomic DNA to be labeled.
9. 0.5 M Ethylenediaminetetraacetic Acid (EDTA).
10. 1% agarose gel (small).
11. 100 bp ladder.
12. Thermocycler or water bath set to  $15^{\circ}\text{C}$ .

## **2.3. Purification of Labeled Probes**

1. Sephadex G-50 (Sigma-Molecular biology grade).
2. Glass wool (Fisher).
3. 1 ml Tuberculin syringe.
4. 15 ml Corex tube.
5. 1 $\times$  TE: 10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0). Make from 10 $\times$  stock.

## **2.4. FISH**

1. 70%, 90%, 100% Ethanol. Keep one set at room temp and another set at  $-20^{\circ}\text{C}$ .
2. 20 $\times$  SSC: 3 M NaCl, 0.3 M  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$  (trisodium citrate 2 hydrate), pH 7.0. Also make 2 $\times$  dilution. Store both at room temperature.
3. 70% Formamide in 2 $\times$  SSC (10 ml): 7 ml deionized formamide, 1 ml 20 $\times$  SSC, 2 ml  $\text{ddH}_2\text{O}$ . Store at  $4^{\circ}\text{C}$ .
4. Deionized Formamide (Amersham).
5. 50% Dextran sulfate: 5 g. Dextran sulfate in 10 ml  $\text{ddH}_2\text{O}$ . Viscous. Aliquot in 1 ml tubes and store at  $-20^{\circ}\text{C}$ .
6. Directly or indirectly labeled DNA probes.
7. Rubber cement.

8. 22 × 22 and 22 × 40 mm glass coverslips.
9. Moist Chamber: Tupperware style container, lined with moist towel, and covered with aluminum foil.
10. Coplin Jar.

## **2.5. FISH Detection**

### *2.5.1. Direct Detection*

1. 2× SSC: Dilute from 20× (see Subheading 2.4).
2. 1× PBS: 0.13 M NaCl, 0.007 M Na<sub>2</sub>HPO<sub>4</sub>, 0.003 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4 (make 10× stock). Store at room temperature.
3. Vectashield Mounting Medium: Supplemented with 4', 6-diamidino-2-phenylindole (DAPI) or Propidium Iodide (PI) (Vector Laboratories Inc).

### *2.5.2. Indirect Detection/ Antibody Layering*

1. 2× SSC: Dilute from 20× (see Subheading 2.4).
2. 1× PBS: 0.13 M NaCl, 0.007 M Na<sub>2</sub>HPO<sub>4</sub>, 0.003 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4 (make 10× stock). Store at room temperature.
3. Secondary antibodies: An exemplary set of antibodies that can be used in series for indirect signal amplification is outlined in Subheading 3 (potential sources: Invitrogen, Roche, Jackson ImmunoResearch, etc.) (also see Note 15).
4. 1× TNT: 0.1 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.5. Make from 10× stock.
5. Vectashield Mounting Medium: Supplemented with DAPI or PI (Vector Laboratories Inc), 5× TNB: 0.5 M Tris-HCl (pH 7.5), 0.75 M NaCl, 2.5% (w/v) Blocking Reagent (Roche). Store at -20°C.
6. Epifluorescence microscope with appropriate filters and image acquisition hardware.
7. Coplin Jars.

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## **3. Methods**

FISH in rice includes two major steps: first, preparation of quality slides containing either meiotically or mitotically derived chromosome spreads and second, the actual probe hybridization and detection steps. The importance of obtaining quality chromosome spreads for producing robust FISH results is often overlooked. For this reason we provide an in-depth description of how to obtain either mitotic or meiotic chromosomes from their respective tissue sources. Tissue collection and chromosome preparation may take several weeks before satisfactory slides are produced. The entire FISH procedure however can be completed in two separate days. The first day consists of probe labeling, denaturing of the probe and target (chromosome) DNA, and an overnight incubation to allow for probe-target heteroduplex formation.

We prefer to use nick translation to label DNA probes. A major advantage of using nick-translation is that the average size of the labeled DNA fragments can be readily controlled. Generally any type of DNA sequence can be used for labeling, including genomic DNA, PCR-amplified DNA fragments, plasmid clones, and BAC clones. However, the PCR fragments or inserts or plasmid clones should be at least 200 bp long. DNA with initial fragments of <200 bp often do not generate good probes.

### **3.1. Preparation of Somatic Chromosomes from Rice Root Tips**

#### *3.1.1. Collection of Root Meristem Tissue for Chromosome Preparations*

Root Tips Harvested  
from Germinating Seeds  
of *Oryza sativa*

1. Place 25–50 seeds on moist filter paper in a Petri dish and incubate at room temperature or slightly above (20–30°C). Setting them on the top of a warm incubator works well (see Note 1).
2. Make sure that filter paper does not dry out over the course of the germination. Change filter paper after a few days.
3. The seeds should germinate in 2–5 days.
4. Harvest the root tips when they are approximately 3–4 cm long.

Root Tips Harvested from  
Potted Plants (See Note 2)

1. Plant an appropriate number of seeds (5–10) in 4 or 6 in. pots. Field soil is the best planting medium for long-term rice growth. Artificial soil can be used if only growing the rice as an immediate source for roots tips and/or short-term use.
2. When roots start to emerge from the bottom of the container, the plants are ready for root sampling.
3. Remove plants from pots by inverting the containers.
4. Using a scissors or forceps, sample actively growing root tips. Healthy, actively growing root tips will be mostly pasty white in color with a slight yellowing toward the tip. Both primary and secondary roots are good sources for mitotic chromosomes.
5. Collect root tips into a container filled with ddH<sub>2</sub>O on ice. Keep the container on ice until sampling is complete.
6. When collection has finished, promptly start the root tip treatment procedures described at the beginning of this section under Subheading 3.1.

#### *3.1.2. Pretreatment of Root Tips and Slide Preparation*

1. Harvest fresh roots 3–4 cm long from potted plants or germinating seedlings as described above.
2. Pretreat the roots in 2 mM 8-hydroxyquinoline at room temperature for 2–4 h to accumulate metaphase chromosome. This time varies depending on species.
3. Fix the roots with fixation solution for 24 h at room temperature and then keep at –20°C. The specimens can be stored in the fixative at –20°C for several months.

4. Wash the roots two times with distilled water for 5 min each (see Note 3).
5. Carefully dissect the meristem tissue away from the rest of the root and place it in a 1.5 ml microfuge tube containing 50  $\mu$ l of digestion cocktail.
6. Incubate at 37°C in water bath for 30–60 min (see Note 4).
7. Carefully remove the digestion cocktail with a pipette. Replace the cocktail with 100  $\mu$ l ddH<sub>2</sub>O and leave tube on ice for 2 min. Repeat this wash procedure two more times.
8. Fix the root tips again by replacing the ddH<sub>2</sub>O with 200  $\mu$ l of fixation solution. Place the sample at –20°C for at least 30 min. Samples can be stored like this for several days if needed.
9. Using a pipette with a cut pipette tip, transfer one digested root tip to an ice-cold and clean microscope slide. Leave approximately 20–30  $\mu$ l of fixation solution surrounding the specimen on the slide.
10. Using a forceps or a pair of probes, quickly macerate the specimen in the fixation solution. Make sure that the fixative does not evaporate. Add more drops if necessary.
11. Upon completely macerating the sample, apply an additional 20  $\mu$ l of the fixation solution to flank each side of the specimen area.
12. Gently heat the bottom of the slide over an alcohol burner. While doing so, briefly expose the specimen and fixation solution to the flame by slightly tilting the slide into the flame. Only expose the sample to the flame long enough for the solution to ignite.
13. Pull the slide away from the flame and allow the flame to extinguish itself by burning up the rest of the fixative. Steps 12 and 13 should not take more than about 5 s.
14. Let the slide dry and cool at room temperature.
15. Slides can be used immediately for FISH. Alternatively, prepared slides can be stored, dry and without a coverslip, in slide box at room temperature or at 4°C.

### **3.2. Preparation of Meiotic Chromosomes at Pachytene Stage from Rice Anthers**

#### **3.2.1. Panicles Containing Anthers at Pachytene Stage of Meiosis**

1. Rice panicles should be harvested from actively growing plants during the booting stage or prior to their emergence from the leaf sheath (see Note 5).
2. Remove the entire panicle from the boot by peeling away the sheath leaves and breaking the panicle away from the stem.
3. Immerse the panicle in fixation solution for 1 day at room temperature, and then store it at –20°C until use.
4. (Optional) It has been suggested that using a vacuum to infiltrate the fixative into the tissue provides superior fixation results. Some members of our lab have found merit in this.

**3.2.2. Preparation of  
Pachytene Chromosome  
Slides from Collected  
Anthers**

1. Remove a panicle from the fixation solution. Remove one floret and place it on a clean microscope slide. Place one drop of fixation solution on specimen to prevent from drying. The rest of the panicle can be replaced back into the fixation solution (see Note 6).
2. Carefully dissect away the anthers from the glume by cutting the filaments attached to the anthers. Do not let the anthers dry.
3. Use a fine needle to gently but thoroughly smash the anther to release the meiocytes. Place one drop of 1% acetocarmine on the slide and mix with cell suspension.
4. Remove extra tissue debris and cover the solution with an 18 × 18 mm or 22 × 22 mm coverslip.
5. Briefly heat the slide using an alcohol burner. Remove excess acetocarmine, by adding one or two drops of 45% acetic acid along one side of coverslip and use a piece of filter paper to wick the fluid from the other side. This will destain the acetocarmine-stained cells.
6. Gently heat the slide using an alcohol burner to just short of the boiling point. It should feel uncomfortably warm to the touch, but not so hot as to boil the solution.
7. Quickly flip the slide upside down and lay it on a piece of filter paper. Gently apply hand pressure to help flatten and spread the cells on the slide.
8. Store the slides at -80°C.

**3.3. Probe Labeling  
(See Note 7)**

1. Prepare the following reaction solution:

<b>Nick Translation MasterMix components</b>	<b>μl of reagent</b>
10× Nick Translation Buffer	5
Unlabeled dNTP solution (+A,C,G) (-T)	2
Labeled dNTP solution (fluorescent-direct) (biotin- or digoxigenin-indirect)	2
Plasmid or Genomic DNA (~1 microgram)	X
DNA Polymerase I	X (12 units)
DNase I (diluted)* (see Note 8)	1
ddH <sub>2</sub> O	X
Total volume	50

2. Gently mix after each step of adding solutions and spin down at the end.
3. Incubate reaction solution in water bath or thermocycler set to 15°C for 1.5 h.
4. Temporarily stop the reaction by placing the tube on ice.

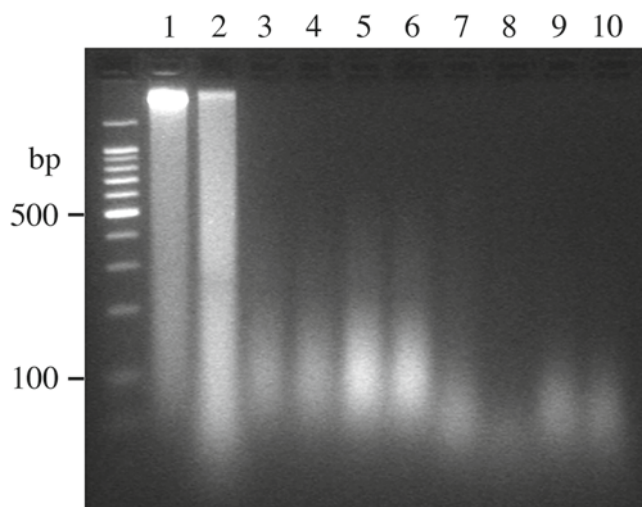


Fig. 1. Gel electrophoresis of nick translated BAC DNA. In each lane, approximately 1  $\mu$ g of DNA from a BAC clone was nick translated. Concentrations of DNase I in nick reactions decrease from left to right across the lanes. Lanes 1 and 2 are considered underdigested; lanes 3–6 are perfectly digested and lanes 7–10 are overdigested.

5. Check the size of the labeled products by running a 4  $\mu$ l aliquot on a 2% agarose gel next to a 100 bp ladder (see Note 9).
6. After staining the gel with ethidium bromide for 10 min, view the DNA size under UV light. If the smear from the product is less than 750 bp then the probe is suitable for FISH and you should go directly to step 8 (see Fig. 1).
7. If the upper limit of the size range is significantly larger than 750 bp (>1 kb), add more DNase I (1–3  $\mu$ l), place the tube at 15°C for another 20–40 min, and then run another 2% agarose gel to check the DNA size.
8. Add 5  $\mu$ l of 0.2 mM EDTA to stop the reaction. At this point, the probe can either be further purified (see Subheading 3.4) or used directly for FISH.

### 3.4. Purification of Labeled Probes (Optional)

1. Plug the bottom of a 1 ml tuberculin syringe with siliconized glass wool.
2. Fill the syringe with Sephadex G-50 up to the top using a sterilized Pasteur pipette.
3. Place the sephadex-filled syringe into a 15 ml Corex tube.
4. Centrifuge at 1,500rpm ( $300\times g$ ) for 4 min to pack down the sephadex.
5. Repeat steps 2–4 so that the packed sephadex column has a total volume of about 0.9 ml.
6. Wash the column twice with 55  $\mu$ l of 1 $\times$  TE and centrifuge at 1,500 rpm for 4 min each.



7. Place a sterilized 1.5 ml tube on the bottom of the 15 ml Corex tube; insert the packed and washed syringe into the Corex tube such that the tip of the syringe is fitted into the 1.5 ml tube. Load the nick translation product (55  $\mu$ l, with stop buffer) and centrifuge at 1,500 rpm for 4 min.
8. The labeled probe DNA is collected in the 1.5 ml tube.

### **3.5. Hybridization of the Probe to Chromosomes**

#### **3.5.1. Target/Chromosome Denaturation**

1. Remove the coverslips from the frozen slides (stored in a  $-80^{\circ}\text{C}$  freezer) with a razor blade by catching an edge of the razor blade under a corner of the coverslip and smartly lifting up (see Note 10).
2. Immediately immerse the slides in an ethanol series (70%, 95%, 100% ethanol) for 5 min each at room temperature. Allow slide to completely dry for 5 min on bench.
3. Add 100  $\mu$ l of the 70% formamide in  $2\times$  SSC solution on the dried slide and cover the slide with a  $22\times 40$  mm coverslip. Denature slide on a hot plate set to  $80^{\circ}\text{C}$  for approximately 60–90 s. Do not exceed 2 min.
4. Let coverslips fall off and immediately immerse into a cold ethanol series (70%, 90%, and 100%) 5 min each at  $-20^{\circ}\text{C}$  and air-dry the slide.

#### **3.5.2. Probe Mix and Denaturation**

1. Prepare the hybridization mixture as follows (see Note 11):

<b>FISH MasterMix components</b>	<b><math>\mu</math>l of reagent</b>
Deionized Formamide	5
50% Dextran Sulfate	2
$20\times$ SSC	1
Indirect or direct labeled probe	1–2
Total volume	$\sim 10/\text{slide}$

2. Denature this mixture by placing sample in an  $80^{\circ}\text{C}$  heat block for 5 min. After denaturation, briefly centrifuge and immerse tube into an ice bath.

#### **3.5.3. Probe/Target Hybridization**

1. Apply 10  $\mu$ l of hybridization mixture to the slide and cover with an  $18\times 18$  mm or  $22\times 22$  mm coverslip. Seal the coverslip by applying a line of rubber cement around the edges and place the slide in a moist chamber (see Note 12).
2. Incubate the wet chamber at  $37^{\circ}\text{C}$  for a minimum of 8 h or overnight. Slides can be kept in a sealed moist chamber for 3–4 days.

### **3.6. Detection of Directly Labeled DNA Probes**

1. Using a forceps, carefully peel off the rubber cement without allowing the coverslip to move. Immerse the slides, still with coverslip, in a coplin jar containing  $2\times$  SSC. Slow shaking of the coplin jar will help the coverslips fall from the slides.

- Place slides in a new coplin jar and wash the slides with the following steps (see Note 13):

2× SSC, room temperature	5 min
2× SSC, 42°C	10 min
2× SSC, room temperature	5 min
1× PBS, room temperature	5 min

- Shake off excess PBS and mount the coverslip by placing one drop (approximately 20 µl) of an antifade-mounting medium (Vectashield) supplemented with a counterstain such as DAPI or PI (see Note 14).
- The slide can now be viewed using fluorescent microscopy. Store the slide in a dark box at -20°C. Slides stored under these conditions will be stable for several months.

### **3.7. Detection of Two Indirectly Labeled DNA Probes with a Single Layer of Antibodies**

- Employ the stringency washes as described in Subheading 3.6.
- Prepare master mix for detecting two labeled probes as follows:

Detection MasterMix components	µl of reagent
5× TNB Buffer	20
ddH <sub>2</sub> O	80
Rhodamine anti-digoxigenin (see Note 15)	1
FITC (Strept)Avidin (see Note 15)	1
Total volume	~100/slide

- Place 100 µl of antibody detection mix on the slide and cover with a 22×40 mm coverslip. Parafilm cut to size can also be used to cover the antibody solution on the slide.
- Place the slide in a sealed moist chamber and incubate at 37°C for 30–60 min.
- Remove the coverslip by tilting the slide and carefully shaking it off.
- Wash the slides using the following steps:

1× TNT	5 min, room temperature (repeat 2×)
1× PBS	5 min, room temperature

- Shake off excess 1× PBS and mount the coverslip by placing one drop (approximately 20 µl) of an antifade mounting medium (Vectashield) supplemented with a counterstain such as DAPI or PI onto the target area. Lay a single 18×18 mm or

22×22 mm coverslip over the solution and seat it using light hand pressure (see Note 14).

8. The slide can now be viewed using fluorescent microscopy. Store the slide in a dark box at  $-20^{\circ}\text{C}$ . Slides stored under these conditions will be stable for several months.

**3.8. Detection and Signal Amplification of Two Indirectly Labeled DNA Probes Using Multiple Layers of Antibodies**

1. Employ the stringency washes outlined in Subheading 3.6.
2. Prepare *layer 1* master mix for detecting two probes:

<b>Multilayer detection MasterMix components: Layer 1</b>		<b>μl of reagent</b>
5× TNB Buffer		20
ddH <sub>2</sub> O		80
Mouse anti-digoxigenin (see Note 15)		1
FITC streptavidin (see Note 15)		1
Total volume		~100/slide

3. Place 100 μl of antibody detection mix on the slide and cover with a 22×40 mm coverslip. Parafilm cut to size can also be used to cover the antibody solution on the slide.
4. Place the slide in a moist chamber and incubate at  $37^{\circ}\text{C}$  for 30–60 min.
5. Remove the coverslip by tilting the slide and carefully shaking it off.
6. Place the slide in a coplin jar and wash in 1× TNT for 5 min. Repeat this wash two more times for a total of three washes in 1× TNT.
7. Decant 1× TNT from coplin jar and replace it with 1× PBS. Rinse for 3 min.
8. Prepare *Layer 2* master mix for detecting two probes (see Note 16):

<b>Multilayer detection MasterMix components: Layer 2</b>		<b>μl of reagent</b>
5× TNB buffer		20
ddH <sub>2</sub> O		80
Goat anti-streptavidin conjugated with biotin (see Note 15)		1
Total volume		~100/slide

9. Repeat steps 3–7.

10. Prepare *Layer 3* master mix for detecting two probes:

Multilayer detection MasterMix components: Layer 3	$\mu\text{l}$ of reagent
5 $\times$ TNB Buffer	20
ddH <sub>2</sub> O	80
FITC streptavidin (see Note 15)	1
Rhodamine (or Texas red) anti-mouse (see Note 15)	1
Total volume	~100/slide

11. Repeat steps 3–7.
12. Shake off excess 1 $\times$  PBS and mount the coverslip by placing one drop (approximately 20  $\mu\text{l}$ ) of an antifade mounting medium (Vectashield) supplemented with a counterstain such as DAPI or PI onto the target area. Lay a single 18 $\times$ 18 mm or 22 $\times$ 22 mm coverslip over the solution and seat it using light hand pressure (see Note 14).
13. The slide can now be viewed using fluorescent microscopy. Store the slide in a dark box at  $-20^{\circ}\text{C}$ . Slides stored under these conditions will be stable for several months.

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## 4. Notes

1. Seeds of wild rice (wild *Oryza* species) species generally take longer to germinate, require a presterilization step, and can also have a lower germination rate. However, seeds of cultivated rice (*Oryza sativa*) should readily germinate on moist/sterile filter paper without further manipulation.
2. Root samples from actively growing plants provide good source material for somatic chromosome preparation. Collect sample tissue from plants that are actively growing and whose roots have not become brown and pot bound. Light pruning of roots and repotting into larger pots can entice older plants to produce new root growth.
3. Most preparations can be readily viewed under phase contrast without staining. If a level of chromosome staining is preferred, we suggest using acetocarmine to stain the root tips prior to slide preparation.
4. The digestion time varies depending on the species and size of the root tip samples. Typically, the root tips are sufficiently digested when upon flicking the tubes, you can observe pieces of root material falling off the otherwise intact root tip.

5. Generally, once panicles have emerged from the leaf sheath meiosis is complete and the materials will no longer yield meiotic chromosomes at pachytene stage. The initiation of meiotic division varies depending on growth conditions and the time of harvest. Initially, panicles at different stages of booting should be sampled to determine optimal timing under your conditions.
6. Because the stage of floret development on a given panicle is variable, different sizes of florets should be checked for the proper stage of meiotic division. Typically slides should be prepared from the smallest florets first and checked under a microscope for the presence of pachytene chromosomes. Based on the cell morphology and stage of meiosis observed, you may need to target slightly more or less developed florets on a given panicle.
7. DNA can be labeled using a “direct labeling” approach in which the DNA itself is labeled with fluorescence-tagged dUTPs such as Texas red-dUTPs or FITC-dUTPs, or using an “indirect labeling” approach in which the DNA is labeled with a nonfluorescent tag using biotin-dUTP and digoxigenin-dUTP. In the latter case the primary DNA tag is subsequently detected with a fluorescently labeled secondary antibody. For FISH, the labeled DNA fragments used in hybridization need to be between ~100 and 500 bp long; otherwise, relatively high levels of background becomes visible on the slide, and the hybridization signal itself becomes less punctuated.
8. DNase I can be purchased as a powder and reconstituted using the DNase I stock buffer. The stock solution needs to be further diluted using the DNase I dilution buffer to reduce the enzyme’s activity. Adjust the concentration of DNase I in the labeling reaction to control the final size of the DNA product such that the average size is 100–500 bp. Since fluorescence tags are light sensitive, all the steps requiring handling of them should be completed in the dark.
9. Next to FISH, the best way to confirm the quality of your labeled product is to check the size of the final nick translation product by running a small agarose gel. Although we introduce 100–500 bp as an optimum size range, a slightly larger size range (100–1,000 bp) will probably work fine. The random priming technique can also be used for labeling. However, since the size of the probe is relatively easier to control by nick translation, we prefer to use the nick translation technique. If you plan on doing only a few FISH experiments you may save time by purchasing one of the many commercial nick translation kits available. However, we often find the consistency of the labeling results of these kits to be variable.

10. If the slides were made using the flame dry method as described above, then steps 1 and 2 of "Target/Chromosome denaturation" can be skipped.
11. If using more than two probes, combine them (0.5–1  $\mu\text{l}$ /slide) into one tube and dry them down in a vacuum centrifuge prior to adding the rest of the ingredients. 50% dextran sulfate is thick, viscous, and best pipetted slowly and with a cut pipette tip. Mix the probe mixture well using a pipette. For probing a larger target area the master mix can be scaled to 20  $\mu\text{l}$ .
12. Plastic boxes with tight closing lids work well as moist chambers (e.g., Tupperware style boxes). Line the bottom of the box with several layers of clean filter paper. Suspend the slides over the filter paper by placing two rods or scoopulas in parallel in the box to set your slides on. Keep the filter paper moist.
13. Protect the slides from light during the washes. You may increase the temperature and time of the 2 $\times$  SSC wash if you want to reduce background. Higher stringency washes can be achieved by completing a 10-min wash in a solution of 50% formamide in 2 $\times$  SSC at 42°C; however, the washing series outlined above works fine for the majority of FISH experiments.
14. The slide can be stained prior to antifade mounting in a solution of 1  $\mu\text{g}/\text{ml}$  of DAPI in 1 $\times$  PBS for 15 min followed by two washes in 1 $\times$  PBS. Intuitively, if the probe is labeled with rhodamine or a rhodamine analog, do not use propidium iodide as a counterstain since both the probe and counterstain will fluoresce red rendering the probe signal indistinguishable.
15. Many different antibodies conjugated with fluorophors are commercially available and are too numerous to list here. We suggest choosing fluorophores with excitation/emission values that most closely coincide with the capabilities of your microscope filters. In our hands both avidin and streptavidin work well for detecting biotin-labeled probes. Fluorescent antibody conjugates should be used at the manufacturer's recommended concentration for in situ detection of probes. Typically a 1:10 to 1:20 dilution (in 1 $\times$  PBS) is acceptable for our working stocks.
16. Our procedure for signal amplification only includes two layers of antibodies for detection of digoxigenin-labeled probes, while our detection of the biotin-labeled probes uses three layers. In our hands, this scheme yields robust and repeatable results; however, this is just one example and experimentation with other layering schemes may be required to detect recalcitrant probes. Finally, we find that using more than three layers to amplify a signal results in elevated levels of background noise and thus applying more than three layers is generally not recommended.

## Acknowledgments

Cytogenetic studies of *Oryza* species in our lab have been supported by grant 2006-35604-16649 from the US Department of Agriculture Cooperative State Research, Education, and Extension Service (CSREES) and grant DBI-0603927 from the National Science Foundation.

## References

1. Chen MS, Presting G, Barbazuk WB, Goicoechea JL, Blackmon B, Fang FC, Kim H, Frisch D, Yu YS, Sun SH, Higingbottom S, Phimphilai J, Phimphilai D, Thurmond S, Gaudette B, Li P, Liu JD, Hatfield J, Main D, Farrar K, Henderson C, Barnett L, Costa R, Williams B, Walser S, Atkins M, Hall C, Budiman MA, Tomkins JP, Luo MZ, Bancroft I, Salse J, Regad F, Mohapatra T, Singh NK, Tyagi AK, Soderlund C, Dean RA, Wing RA (2002) An integrated physical and genetic map of the rice genome. *Plant Cell* 14:537–545
2. Jiang JM, Gill BS, Wang GL, Ronald PC, Ward DC (1995) Metaphase and interphase fluorescence in situ hybridization mapping of the rice genome with bacterial artificial chromosomes. *Proc Natl Acad Sci USA* 92:4487–4491
3. Cheng Z, Presting GG, Buell CR, Wing RA, Jiang JM (2001) High-resolution pachytene chromosome mapping of bacterial artificial chromosomes anchored by genetic markers reveals the centromere location and the distribution of genetic recombination along chromosome 10 of rice. *Genetics* 157:1749–1757
4. Kurata N, Omura T, Iwata N (1981) Studies on centromere, chromomere and nucleolus in pachytene nuclei of rice, *Oryza sativa*, microsporocytes. *Cytologia* 46:791–800
5. Khush GS, Singh RJ, Sur SC, Librojo AL (1984) Primary trisomics of rice: origin, morphology, cytology and use in linkage mapping. *Genetics* 107:141–163
6. Chung MC, Wu HK (1987) Karyotype analysis of 'IR36' and two trisomic lines of rice. *Bot Bull Acad Sin* 28:289–304
7. Cheng Z, Buell CR, Wing RA, Gu M, Jiang JM (2001) Toward a cytological characterization of the rice genome. *Genome Res* 11:2133–2141
8. Kao FI, Cheng YY, Chow TY, Chen HH, Liu SM, Cheng CH, Chung MC (2006) An integrated map of *Oryza sativa* L. chromosome 5. *Theor Appl Genet* 112:891–902
9. Cheng ZK, Buell CR, Wing RA, Jiang JM (2002) Resolution of fluorescence in-situ hybridization mapping on rice mitotic pro-metaphase chromosomes, meiotic pachytene chromosomes and extended DNA fibers. *Chromosome Res* 10:379–387
10. Dong F, Jiang JM (1998) Non-Rabl patterns of centromere and telomere distribution in the interphase nuclei of plant cells. *Chromosome Res* 6:551–558
11. Fukui K, Ohmido N, Khush GS (1994) Variability in rDNA loci in the genus *Oryza* detected through fluorescence in-situ hybridization. *Theor Appl Genet* 87:893–899
12. Ohmido N, Fukui K (1997) Visual verification of close disposition between a rice A genome-specific DNA sequence (TrsA) and the telomere sequence. *Plant Mol Biol* 35:963–968
13. Ohmido N, Kijima K, Akiyama Y, de Jong JH, Fukui K (2000) Quantification of total genomic DNA and selected repetitive sequences reveals concurrent changes in different DNA families in *indica* and *japonica* rice. *Mol Gen Genet* 263:388–394
14. Cheng Z, Stupar RM, Gu M, Jiang JM (2001) A tandemly repeated DNA sequence is associated with both knob-like heterochromatin and a highly decondensed structure in the meiotic pachytene chromosomes of rice. *Chromosoma* 110:24–31



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Rice Protocols

Yang, Y. (Ed.)

2013, XII, 335 p., Hardcover

ISBN: 978-1-62703-193-6

A product of Humana Press