Chapter 14

RNA FISH, DNA FISH and Chromosome Painting of Chicken Oocytes

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Abstract

Fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique. It identifies the location of DNA loci and RNAs, including nascent RNAs in the process of being transcribed, within individual cells. Great advances in fluorescent dye technology and technique sensitivity, combined with developments in light microscopy and imaging software have made it widely accessible and have expanded the range of applications in basic research as well as in diagnostics. Being able to perform RNA hybridization, DNA hybridization, and protein immunofluorescence consecutively on the same sample is an invaluable tool to study RNA expression in relation to their gene loci and to map RNA and DNA in relation to nuclear or cellular structures. This has contributed to enormous progress in understanding basal mechanisms of male and female meiosis in different animal model systems. In this chapter we describe in detail the protocols for FISH based techniques applied to study gene expression dynamics and nuclear architecture of chicken oocytes during meiotic prophase I. These techniques can be easily performed in any molecular and cell biology laboratory and be adapted to different systems and to different phases of gametogenesis.

Key words Chicken oocyte, Meiosis, Fluorescence in situ hybridization, Chromosome painting

1 Introduction

Originally developed in 1969 by Gall and Pardue [1] to detect the position of DNA sequences within the cell, the in situ hybridization procedure brought cytogenetics to the molecular era. Today most in situ hybridization protocols make use of fluorescence for target detection and therefore it is referred to as Fluorescence In Situ Hybridization or FISH [2]. The basic technique implies the labeling of a DNA probe and its hybridization to cells fixed on a slide. The target sequence is visualized as a fluorescent signal within the cell. Since its development, FISH has greatly improved its sensitivity, specificity, and resolution power. This, combined with technological advancements bringing a wide range of detection methods, multiplex-detection, sensitive quantitative measurements and the development of microfluidic arrays, has transformed

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molecular cytology. FISH has now evolved into a plethora of versatile methods that allow the examination of DNA loci (DNA FISH) as well as RNAs (RNA FISH) [3]. The range of applications in research and diagnostics continues to expand and now includes karyotyping, genotyping, cancer diagnostics, genome evolution, and gene expression studies [4]. Chromosome painting, one of the first applications of DNA FISH, identifies whole chromosomes using chromosome-specific DNA libraries as probes [5]. Now each chromosome can be labeled with a different fluorophore to generate a spectral karyotyping [6].

The possibility to combine RNA FISH with DNA FISH and protein immunofluorescence means that we can now visualize RNA and DNA in situ in direct relation with endogenous proteins, in particular proteins that define specific cytoplasmic or nuclear cell compartments. This has made FISH an invaluable tool in the field of reproductive science and in particular to study oogenesis and spermatogenesis. For example, it has been instrumental in understanding fundamental processes in meiosis, such as the mechanism of meiotic sex chromosome inactivation (MSCI) and its evolution [7–10].

Many labs are using RNA/DNA FISH to investigate the topographic relations between DNA, nascent RNA and chromatin-associated proteins in late diplotene oocyte from species with chromosomes in the lampbrush form. The high resolution power of this system has made it into an ideal model to study the epigenetic mechanisms of transcriptional regulation [11]. In human, multicolor FISH and more recently comparative genomic hybridization (CGH) and array CGH are the basis for prenatal diagnosis. The routine use of these techniques to screen embryos generated by in vitro fertilization are starting to shed light on the types of maternal and paternal meiotic errors that lead to aneuploidy [12].

In this chapter we describe the FISH protocols we use to detect nascent RNA transcripts and DNA loci, or whole chromosomes, on spreads from chicken ovaries containing oocytes in early stages of meiotic prophase I. We also discuss how to combine these labeling techniques with protein immunofluorescence. These protocols have been adapted from methods developed for the study of mouse meiosis [13] and can be further adapted to be used in other animal models.

2 Materials

2.1 Preparation of BAC Probes

1. LB agar.
2. LB broth.
3. 25 mg/ml chloramphenicol.
4. P1 solution: 15 mM Tris–HCl pH 8, 10 mM EDTA pH 8, 100 μg/ml RNase A (ice-cold).
5. P2 solution: 0.2 M NaOH, 1% SDS.

6. P3 solution: 3 M potassium acetate (KCH_3COO) pH 5.5 (ice-cold).
7. Ice-cold isopropanol.
8. 1× TE pH 7.5: 10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.
9. 10 mg/ml RNase A.
10. Phenol.
11. (Phenol–chloroform)–isoamyl alcohol 24:1.
12. Chloroform–isoamyl alcohol 24:1.
13. 100% Ethanol (EtOH).
14. 70% EtOH.
15. 5 M NaCl.
16. Commercial kits for alternative DNA extraction: Norgen BAC DNA MiniPrep kit (#18000), or Sigma PhasePrep BAC DNA kit (#NA0100).
17. DIG-Nick translation mix (Roche, 11745816910).
18. BioNick™ DNA labeling System (Invitrogen, 18247-015).
19. 10 mg/ml salmon sperm DNA.
20. Deionized formamide.
21. 37 °C incubator.
22. 37 °C shaking incubator.
23. Centrifuge.
24. Refrigerated microcentrifuge.
25. 15 °C water bath.
26. Agarose.
27. Gel electrophoresis system.

2.2 Labeled Chicken Chromosome Paint Probes

Specific chicken chromosome paints labeled with Biotin-16-dUTP or DIG-11-dUTP can be obtained from FARMACHROM (*see* **Note 1**).

2.3 Preparation of Oocyte Spreads for RNA FISH

1. Ice-cold PBS buffer (Phosphate-buffered saline).
2. Ice-cold CSK buffer: 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES, adjusted to pH 6.8 and filtered; it can be aliquoted and stored at 4 °C for short term storage or –20 °C for long term storage—Supplements to be added fresh: 0.5% Triton X-100, 1 mM EGTA, 2 mM vanadyl ribonucleoside.
3. Ice-cold 4% paraformaldehyde (PFA) in PBS pH 7 (made fresh and filtered).
4. Ice-cold EtOH series: 70%, 80%, 95%, 100%.
5. 6 cm petri dish.
6. Scalpel blades.

7. Superfrost® slides.
8. Ice-cold platform (glass plate on top of small ice-bucket).
9. Coplin jars.

2.4 Alternative Preparation of Oocyte Spreads

1. PBS buffer.
2. Cell suspension buffer: 9% sucrose, 0.05% Triton X-100 in PBS, adjusted to pH 7; it can be aliquoted and stored at 4 °C for short term storage or −20 °C for long term storage.
3. 2% formaldehyde (FA), 0.02% SDS in PBS, adjusted to pH 7 (made fresh and filtered).
4. 6 cm petri dish.
5. Scalpel blades.
6. Superfrost® slides.
7. Ice-cold platform (glass plate on top of small ice-bucket).
8. Coplin jars.

2.5 RNA FISH

1. 2× hybridization buffer: 4× SSC, 50% dextran sulfate, 2 mg/ml BSA, 2 mM Vanadyl ribonucleoside.
2. Wash solution 1: 50% formamide, 1× SSC.
3. Wash solution 2: 2× SSC.
4. Equilibration buffer: 4× SSC, 0.1% Tween 20.
5. Blocking buffer: 4× SSC, 4 mg/ml BSA, 0.1% Tween 20.
6. Detection buffer: 4× SSC, 1 mg/ml BSA, 0.1% Tween 20.
7. FITC-conjugated anti-digoxigenin (affinity purified sheep polyclonal antibody, part 90426 of Apoptag® kit (S7111) from Merck-Millipore).
8. Vectashield mounting medium with DAPI (Vector).
9. Coplin jars, coverslips.
10. 85 °C dry block.
11. Shaker.
12. 37 °C incubator.
13. 42 °C incubator.
14. Tip Top vulcanizing solution (Rema) (optional).
15. Humidified chambers: box containing paper towels soaked in 50% formamide, 2× SSC; box containing paper towels soaked in 4× SSC.
16. Refrigerated microfuge.

2.6 DNA FISH/ Chromosome Painting

1. PBS buffer.
2. Pepsin solution: 500 µg/ml pepsin in 10 mM HCl.
3. 4% PFA in PBS pH 7.

4. 2× SSC.
5. 100 µg/ml RNase A in 2× SSC (optional).
6. Denaturation solution: 70% deionized formamide, 30% 2× SSC.
7. Ice-cold EtOH series: 70%, 80%, 95%, 100%.
8. 2× hybridization buffer: 4× SSC, 50% dextran sulfate, 2 mg/ml BSA.
9. Wash solution A: 2× SSC.
10. Wash solution B: 0.1× SSC prewarmed at 60 °C.
11. Equilibration buffer: 4× SSC, 0.1% Tween 20.
12. Blocking buffer: 4× SSC, 5% dry milk.
13. Streptavidin, Alexa Fluor® 555 conjugate (Invitrogen S21381).
14. Biotinylated anti-streptavidin antibody (Goat antibody, Vector BA05000).
15. Vectashield mounting medium with DAPI (Vector).
16. 37 °C water bath (optional).
17. Coplin jars, coverslips.
18. 80 °C and 60 °C incubators.
19. 85 °C dry block.
20. Shaker.
21. 37 °C incubator.
22. 42 °C incubator.
23. Tip Top vulcanizing solution (Rema) (optional).
24. Humidified chambers: box containing paper towels soaked in 50% formamide, 2× SSC; box containing paper towels soaked in 4× SSC.
25. Refrigerated microfuge.

3 Methods

Before starting a FISH experiment on chicken oocytes there are several things to consider that are discussed below.

If analyzing early prophase I, bear in mind that the oocytes enter meiosis in an asynchronous fashion. Before hatching the ovary mostly contains oocytes in pre-leptotene, leptotene, and zygotene phases. Pachytene cells start to be common at hatching and become the predominant population between day 1 and 3. Diplotene cells commonly appear at around day 3 and become the major type by day 6. The synaptonemal complex protein SYCP3 is a good marker to stage the cells up to this point [8], but later diplotene cells need to be identified in other ways (e.g., with markers for the centromere or for cohesins).

In multi-label experiments it is advisable to start with RNA FISH, followed by protein immunofluorescence and finally DNA FISH. This is because RNA can be easily degraded. The only instance immunostaining should be done first is when the protein epitope could be harmed by the RNA FISH protocol. In this case try to preserve the RNA by using RNase free solutions also for immunofluorescence and add an RNase inhibitor, such as RNasin® or RNaseOUT™ to the antibody.

Efficient and reliable hybridization to nascent RNAs and corresponding DNA targets is routinely achieved with the use of double strand DNA probes at least 10 kb long. We generally use BAC DNA probes because longer target sequences yield stronger signal (*see* Subheading 3.1 for DNA preparation), but it is possible to generate the probes by PCR. To this end you can choose to do a long range PCR or multiple PCRs generating fragments of the same size covering around 10 KB of the target sequence. After PCR DNA purification, the long probe or the pool of probes can be labeled following the procedure described for BAC DNA probes in Subheadings 3.2, 3.3 and 3.4. When generating the probe by synthesis, if the target sequence is a nascent RNA transcript, make sure to design a probe that contains both intronic and exonic sequences.

We generally use the same DNA sequence to make probes to detect the RNA and the corresponding DNA locus. One probe is labeled with digoxigenin and the other with biotin. These probes are then indirectly detected using proteins/antibodies conjugated to different fluorochromes.

Probe labeling is achieved by nick-translation, which replaces some of the nucleotides with their labeled analogs. During this process the probe is fragmented to generate a pool of tagged fragments whose size is a function of the reaction time. The optimal range of fragments for both RNA and DNA FISH hybridization is around 200 bp. Long probes do not penetrate the cell nucleus very easily and tend to give a high background.

We routinely analyze the results using a compound fluorescence microscope and digital imaging. We generally avoid confocal microscopy to limit fluorescence bleaching. We score the cells using a high magnification lens, generally a 100× oil immersion objective with high numerical aperture. Nascent RNA targets appear as bright small spots. These are generally close to the corresponding DNA sequence. Nonspecific signals in RNA FISH are generally large and irregular in shape. Remember that RNA FISH targeted to nascent transcripts reflects the real transcription status of the individual cell. It is therefore normal that only a percentage of the oocytes display the signal. It is also advisable to score positive and negative cells under the microscope and only then to proceed to imaging the results.

DNA FISH can also be used as a control of the reliability of the RNA FISH to study gene expression, as probes that work for DNA FISH should also work for RNA FISH.

3.1 BAC DNA Preparation

Inoculate a single colony from a freshly streaked plate into 25 ml of LB medium with the appropriate antibiotic and grow overnight in a 37 °C shaking incubator (*see* **Note 2**).

Proceed to isolate the BAC DNA using a basic alkaline lysis miniprep method as described below.

1. Transfer the culture into two 15 ml tubes and centrifuge for 10 min at 3500 × *g* (best in a swinging bucket centrifuge) at room temperature (RT).
2. Resuspend the pellet in 1 ml of P1 solution by vortexing.
3. Add 1 ml of P2 solution and mix by gently inverting the tubes. Incubate at RT for 5 min (the suspension becomes translucent).
4. Add 1 ml of ice-cold P3 solution and mix by inverting the tubes. Incubate on ice for 5 min (a thick precipitate will form).
5. Aliquot the suspension into two 1.5 ml tubes (about 1.4 ml each) (leaving behind most of the precipitate) and centrifuge in a microfuge at 13,000 rpm (16,200 RCF) for 10 min at 4 °C.
6. Transfer 1.2 ml of supernatant into two 2 ml tubes (avoiding the white precipitate) (*see* **Note 3**).
7. Add 0.6 Volumes (V) of ice-cold isopropanol, mix by inverting the tubes, and centrifuge in a microfuge at max speed for 15 min at 4 °C.
8. Discard the supernatant, add 1 ml of 70% EtOH, mix by inverting the tubes and centrifuge 5 min at max speed.
9. Air-dry the pellet at RT until it turns translucent (avoid over-drying as it makes it difficult to resuspend the DNA), then resuspend it in 120 μl of 1× TE per tube, by tapping the tubes and by incubating them at 37 °C for at least 1 h.
10. Add 1 μl of RNase A to each tube and incubate at 37 °C for at least 1 h (you can leave it overnight).
11. Combine the two tubes (240 μl in total), add 1× TE to have 400 μl total volume and remove RNase A by phenol–chloroform extraction: add 400 μl of phenol, invert the tube several times, centrifuge in microfuge at max speed and transfer upper phase to a new tube. Repeat this step using 400 μl of (phenol–chloroform)–isoamyl alcohol 24:1 and repeat again using 400 μl of chloroform–isoamyl alcohol 24:1. Avoid carryover of solvents.
12. Precipitate the DNA by adding 200 mM NaCl and 2 V of 100% ice-cold EtOH.
13. Wash pellet with 500 μl of 70% EtOH, centrifuge in microfuge at max speed for 5 min.
14. Air-dry the pellet and resuspend it in 32 μl RNase free H_2O (*see* **Note 4**).
15. Measure the DNA concentration using a spectrophotometer (*see* **Note 5**).

3.2 Generating a BAC DNA Probe Using the BioNick™ Labeling System

1. Use 1–2 μg of BAC DNA in 40 μl H_2O (*see* **Note 6**), mix by pipetting.
2. Add 5 μl of dNTP mix from the kit.
3. Add 5 μl of 10× enzyme mix from the kit.
4. Mix well and spin down briefly.
5. Incubate at 15 °C until the probe length is in the range of 200 bp. Check probe length by gel electrophoresis, running an aliquot of the reaction (5 μl) on a 2% agarose gel; if necessary extend the incubation time and run another aliquot on the gel (*see* **Note 7**).
6. Stop the reaction by adding 5 μl of stop buffer from the kit.
7. Store the probe at −20 °C until ready to use.

3.3 Generating a BAC DNA Probe Using the DIG-Nick Translation Mix

1. Use 1–2 μg of BAC DNA in 16 μl H_2O (*see* **Note 6**), mix by pipetting.
2. Add 4 μl of DIG-Mix (5×) from the kit.
3. Mix well and spin down briefly.
4. Incubate at 15 °C until the probe length is in the range of 200 bp. Check probe length by gel electrophoresis, running an aliquot of the reaction (2 μl) on a 2% agarose gel; if necessary extend the incubation time and run another aliquot on the gel (*see* **Note 7**) (Fig. 1).
5. Store the probe at −20 °C until ready to use.

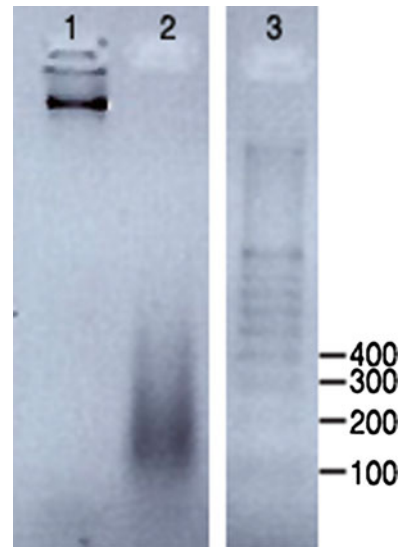

Fig. 1 DIG-labeling of a BAC DNA probe. *Lane 1*, an aliquot (2 μl/32 μl) of a BAC DNA from the alkaline miniprep; *lane 2*, an aliquot (2 μl/20 μl) of the BAC DNA after DIG-labeling showing a smear in the range of 100–300 bp; This smear range is optimal for RNA or DNA hybridization. *Lane 3*, 100 bp ladder

3.4 Preparation of the Probe for Hybridization

Use 1/10 V of the BAC DNA labeled probe generated as described in Subheading 3.2 or 3.3 per slide to be hybridized. Scale up based on number of slides.

1. Transfer 5 μl of biotinylated probe or 2 μl of DIG probe to a 0.5 ml tube and add 1 μl of salmon sperm DNA; adjust the total volume to 9 μl with H_2O (*see* **Note 8**).
2. Precipitate the DNA by adding 3 V of 100% EtOH; store at −80 °C for 10 min then centrifuge at max speed in a microfuge for 15 min at 4 °C.
3. Wash the pellet with 20 μl of 70% EtOH and centrifuge again at max speed for 5 min at 4 °C.
4. Air-dry and resuspend the pellet in 15 μl of formamide by vortexing.
5. To prepare chromosome paint probes *see* **Note 9**.
6. Store at −20 °C, or proceed to use in hybridization: denature the probe at 85 °C for 10 min, add 15 μl of 2× hybridization buffer preheated at 37 °C and incubate at 37 °C for 20 min before placing it onto the slide (*see* **Note 10**).

3.5 RNA FISH

1. Before starting to process the ovary, boil Superfrost® slides in distilled H_2O for 10 min (*see* **Note 11**), air-dry the slides and then place on an ice-cold, horizontal platform.
2. Dissect the left ovary in cold PBS ensuring the connected mesonephros is removed without compromising the surface epithelium of the ovary, as the germ cells are just beneath. From one embryonic or early post-hatching ovary you can generate six to eight spreads (*see* **Note 12**).
3. Transfer the ovary to a 150 μl drop of ice-cold PBS on a small Petri dish (6 cm) and mince it with scalpel blades to obtain a milky suspension.
4. Tilt the Petri dish, add another 300 μl of cold PBS and pipette up and down a few times to maximize the release of germ cells.
5. Wait for 5 min to allow for the debris to sink.
6. Place 70 μl of the suspension on the center of each slide using a micropipette and avoid aspirating the debris. Drop the suspension from a good height (few cm) to allow spreading and leave for 5 min to settle down.
7. Permeabilize the cells by flooding the slide with ice-cold CSK + supplements; leave for 10 min.
8. Drain the liquid, first by gentle aspiration with a micropipette at one end of each slide, then by placing paper towels at one end of the slides and tilting the platform.
9. Fix the cells by flooding the slide with 4% ice-cold PFA and leave for 10 min.

10. Remove the slides from the cold platform and pour off the fixative.
11. Rinse the slides in a Coplin jar filled with ice-cold RNase free H_2O.
12. Transfer to a Coplin jar with ice-cold 70% EtOH for 3 min and proceed to dehydrate via an ethanol series starting with a second wash in 70% EtOH, then 80, 95, and 100% for 3 min each.
13. Air-dry thoroughly at RT (*see* **Note 13**).
14. In the meantime process the DNA probe as described in Subheading 3.4. Be ready to start the probe denaturation step (described in **step 6** of Subheading 3.4) while performing the dehydration of the slides in EtOH (**step 12**).
15. Place the probe from **step 6** in Subheading 3.4 on the center of the slide and add a coverslip avoiding bubble formation (*see* **Note 14**).
16. Put the slides in a humidified chamber and incubate overnight at 37 °C (*see* **Note 15**).
17. On the next day preheat a Coplin jar and stringency wash solutions 1 and 2 at 42 °C.
18. Perform three 5 min washes in the Coplin jar filled with prewarmed wash solution 1 on a shaker at RT. If the coverslip is too adherent to the slide remove it only after the first wash.
19. Perform another three 5 min washes in wash solution 2 at 37 °C with shaking.
20. Transfer slides in equilibration buffer at RT and leave until ready to proceed to the next step.
21. Transfer the slides to a humidified chamber (containing paper towels soaked in 4× SSC) and drop 100 μl of blocking buffer on top; place the coverslip and incubate at 37 °C for 30 min.
22. If the hybridization was performed using a DIG-labeled DNA probe, dilute the FITC-conjugated anti-digoxigenin antibody 1:10 in detection buffer and spin 10 min at 4 °C in a microfuge to remove potential precipitates (*see* **Note 16**).
23. Pour off the blocking buffer from the slides and add 100 μl of the diluted antibody per slide; place the coverslip and incubate in the humidified chamber at 37 °C for 60 min.
24. Remove the coverslip and perform three 5 min washes in equilibration buffer at RT with shaking.
25. Drain the slides; at this point you can proceed immediately to immunostaining and/or, if needed, DNA FISH/chromosome painting, or you can add mounting medium plus DAPI and image. Slides in mounting medium can be stored at −20 °C and processed for antibody staining and/or DNA FISH in the next few days (*see* **Note 17**) (Fig. 2).

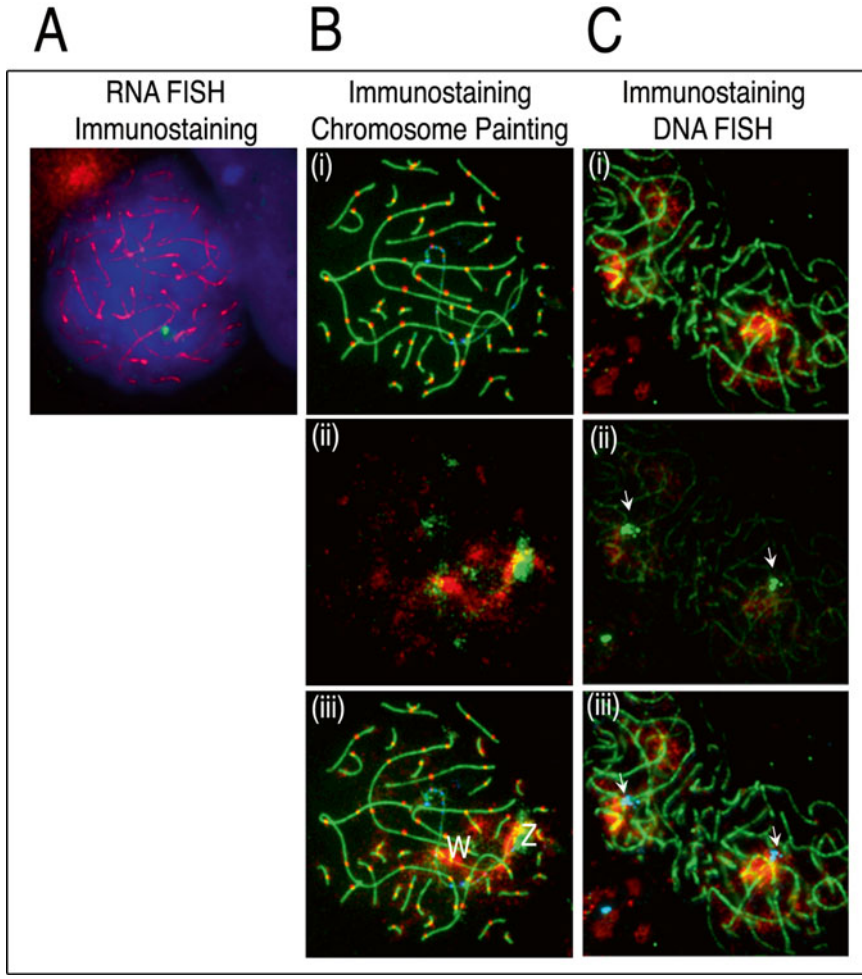

Fig. 2 Oocyte nuclei from 1 day post hatching ovarian spreads. (**A**) Late zygotene/early pachytene cell processed for RNA FISH followed by immunostaining; the RNA FISH signal in *green* identifies nascent transcripts detected with a BAC DNA probe mapping to the Z chromosome. *Red* is the immunofluorescence signal for the SYCP3 protein, which labels the lateral elements of the synaptonemal complex (SC). (**B**) (*i*) Late pachytene cell immunostained for SYCP3 (*green*), MLH1 (*red*) and RPA-32 (*blue*) which label SCs, crossovers and sites of unrepaired double strand breaks, respectively. (*ii*) Same cell from (*i*) reimaged after processing the slide for Z (*green*) and W (*red*) chromosome painting. (*iii*) Composite image from (*i*) plus (*ii*). This late pachytene cell carries over unrepaired double strand breaks on the Z chromosome. (**C**) (*i*) Two pachytene cells Immunostained for SYCP3 (*green*) and H3K9me3 (*red*) which label SCs and heterochromatin blocks, respectively; (*ii*) same cells from (*i*) reimaged after DNA FISH using the BAC DNA probe used in panel **A** (the signal, in *green*, is indicated by *arrows*); (*iii*) Composite image from (*i*) plus (*ii*) where the DNA FISH signal is pseudocolored in *light blue*. The locus detected by the probe is outside the heterochromatin block. Note that the RNA FISH signal in panel **A** is small and tight, while the DNA FISH signal (using the same probe) in panel **C** is made by multiple spots

3.6 Oocyte Spreads for Immuno-fluorescence and DNA FISH/Chromosome Painting

To generate slides with well spread chicken oocytes from embryonic ovaries and 1–2 weeks post-hatch ovaries we use a variation of the protocol used for RNA FISH (*see* **Note 18**). Essentially follow **steps 1–6** in Subheading 3.5 to release the germ cells from the ovary then:

1. Permeabilize the cells by flooding the slide with 800 μl of cell suspension buffer; leave for 10 min.
2. Drain the liquid, first by gentle aspiration at one end of each slide using a micropipette, then by placing paper towels and tilting the slide.
3. Fix the cells by flooding the slides with PBS containing 2% formaldehyde, 0.02% SDS (pH 7) and leave for 1 h (cover the platform with a lid to generate a humidified chamber).
4. Drain the liquid on paper towels by tilting the slides or the all platform; rinse the slides in a Coplin jar filled with H_2O and then let them air-dry upright (*see* **Note 13**).

3.7 DNA FISH and Chromosome Painting

The protocols for DNA FISH and chromosome painting are essentially the same with a minor modification concerning the preparation of the probe, which is addressed in Subheading 3.4. Moreover many of the steps are the same as for RNA FISH.

DNA FISH or chromosome painting can be performed on newly prepared spreads (fresh or after storage). In this case it is important to promote access to chromatin by aging the slides. This can be done by incubating the slides at 60 °C for 2–3 h, or at RT in pepsin solution for 10 min just before starting the protocol.

If DNA FISH or chromosome painting is performed after immunohistochemistry, RNA FISH, or RNA FISH plus immunohistochemistry, the aging step is not necessary; instead, post-fix the slides in 4% PFA for 10 min at RT before starting.

Then:

1. Three 5 min washes in PBS, at RT.
2. One 5 min wash in 2× SSC, at RT (*see* **Note 19**).
3. Incubate 5 min in preheated 2× SSC at 80 °C.
4. Denature the chromatin by incubating the slides in preheated denaturation solution for 3 min at 80 °C.
5. Transfer to a Coplin jar with ice-cold 70% EtOH for 3 min and proceed to dehydrate via an ethanol series starting with a second wash in 70% EtOH, then 80, 95, and 100% for 3 min each.
6. Air-dry thoroughly at RT.
7. In the meantime process the DNA probe as described in Subheading 3.4. Be ready to start the probe denaturation step (described in **step 6** of Subheading 3.4) while performing the dehydration of the slides in EtOH (**step 5**).

8. Place the probe from **step 6** in Subheading 3.4 on the center of the slide and add a coverslip avoiding bubble formation (*see* **Note 14**).
9. Put the slides in a humidified chamber and incubate overnight at 37 °C (*see* **Note 15**).
10. On the next day transfer the slides to a Coplin jar.
11. Perform four 3 min washes in wash solution A at 42 °C (the coverslip should fall off after the first wash, if not, carefully ease it off the slide).
12. Perform four 3 min stringency washes in preheated wash solution B at 60 °C.
13. Wash briefly in equilibration buffer preheated at 37 °C.
14. Drain the slides, transfer them to a humidified chamber (containing paper towels soaked in 4× SSC) and drop 100 μl of blocking buffer on top; place a coverslip and incubate at 37 °C for 30 min.
15. In the meantime prepare substrates and antibodies for the detection steps. To detect the biotinylated probe, dilute the streptavidin–Alexa 555 1:100 in blocking buffer and spin the solution in a microfuge at 10,000 rpm (9,600 RCF) for 10 min at 4 °C to remove any precipitate (*see* **Note 20**).
16. Slide off the coverslip and drain the buffer.
17. Add 50 μl of diluted streptavidin–Alexa 555 on top of each slide, add the coverslip and place the slides back in the humidified chamber at 37 °C for 30 min.
18. Remove the coverslip, transfer the slides to a Coplin jar and do four 3 min washes in equilibration buffer at 37 °C with shaking.
19. Drain the slides, replace the coverslip and observe under a fluorescence microscope if the signal is present. If so, proceed to **step 24**. If the signal is not present, or it is too weak, proceed with amplification **steps 20–23**. If the slides were simultaneously hybridized to a DIG probe, proceed to step 25 now or after the biotin signal amplification (**steps 20–23**).
20. Dilute the biotin conjugated anti-streptavidin antibody 1:100 in blocking buffer and spin the solution in a microfuge at 10,000 rpm for 10 min at 4 °C to remove any precipitate.
21. Add 50 μl of diluted biotin conjugated anti-streptavidin antibody on top of each slide, add the coverslip and place the slides back in the humidified chamber at 37 °C for 30 min.
22. Repeat **step 18** washes.
23. Repeat **steps 16** and **18** to reapply the streptavidin–Alexa 555.
24. Drain the slides and mount it with a drop of mounting medium plus DAPI and place a coverslip. The slides are ready for imaging or can be stored at –20 °C.

25. To reveal the DIG probe dilute the FITC conjugated anti-DIG antibody 1:10 in blocking buffer and spin the solution in a microfuge at 10,000 rpm for 10 min at 4 °C to remove any precipitate.
26. Add 50 μl of diluted FITC-conjugated anti-DIG antibody to each slide, place the coverslip and place the slides back in the humidified chamber at 37 °C for 60 min.
27. Repeat **step 18** washes and proceed to finalize as in **step 24** (Fig. 2).

4 Notes

1. FARMACHROM is a resource center based at the University of Kent, Canterbury, CT2 7NJ, UK, and it is a part of the laboratory of Prof. D. Griffin. Chicken chromosome paints and other avian resources can be requested at the site www.farmachrom.net by filling in a resource request form. The paints are generated and labeled by degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) on flow sorted or microdissected chromosomes [14]. It is possible to ask for unlabeled or labeled paints.
2. A 25 ml culture should yield enough BAC DNA for at least two labeling reactions.
3. Alternatively distribute ~900 μl of the supernatant into three 1.5 ml tubes.
4. Alternatively to this basic protocol you can use commercially available kits for small scale preparation of BAC DNA. We tested the BAC DNA miniPrep kit from Norgen and the PhasePrep BAC DNA kit from Sigma and they are both suitable. The Norgen kit is based on spin column chromatography. Follow the manufacturer's instructions, with some modifications as follows. After spinning the bacterial culture, resuspend pellet in 800 μl of resuspension buffer; scale up next steps of lysate preparation and column binding accordingly. You will use two columns per culture. Wash the DNA bound to the columns with 600 μl of wash solution twice; elute the DNA from each column in 70 μl of elution buffer. Combine the elution from the two columns and extend the manufacturer protocol to include an EtOH precipitation step (add 200 mM NaCl, 2 V of 100% EtOH and spin at max speed in a microfuge for 20 min at 4 °C; also perform a 70% EtOH wash) to get better purification. Resuspend in 32 μl of H_2O.

   The Sigma PhasePrep BAC DNA kit is based on temperature mediated extraction of impurities and phase separation. It consistently produces good quality DNA that is suitable for

labeling without further steps; it is also easily scalable. Follow the manufacturer's instructions for micro scale preparation, scaling up the prep accordingly. After spinning the 25 ml bacterial culture, resuspend pellet in 800 μl of resuspension solution (at this point it is best to transfer the sample into two 2 ml tubes for easy processing) and scale up the next steps accordingly until the nucleic acid precipitation step (**step 5** in their protocol). Resuspend the pellet in 800 μl of elution solution and scale up again the next steps accordingly. Dissolve the purified DNA in 32 μl of H_2O.

5. We use a NanoDrop spectrophotometer for microvolumes. Alternatively you can estimate the concentration by gel electrophoresis.
6. Generally around half of the DNA prepared from the miniprep.
7. The labeling step is very important and therefore it has to be monitored. Electrophoresis is used to visualize the smear produced by nick-translation. Under-labeled probes will appear as high molecular weight smears. These long probes poorly penetrate cells and therefore seldom generate good specific signals while promote nonspecific background in the form of aggregates outside the nuclei. It is safe to check the labeling after 30 min of incubation and decide whether to continue the reaction and for how long. It is possible to speed up the reaction by a short burst at 37 °C. The incubation time is mainly dependent on DNA concentration and purity, so it has to be determined empirically every time a new preparation is made.
8. Many FISH protocols add Cot-1 DNA (the repetitive DNA fraction) to the probe as unlabeled competitor, to neutralize the repetitive DNA present in the probe. We routinely do RNA FISH and DNA FISH using BAC probes without Cot-1 and do not generally have much background problems. At the moment chicken Cot-1 DNA is not commonly available on the market and you have to find your own source, or generate it in your laboratory. If adding Cot-1 DNA, do so at this step before adding the EtOH for the precipitation: typically 3 μg.
9. Labeled chicken chromosome paint probes provided by FARMACHROM are ready to be used for hybridization, so there is no need for the precipitation procedure. Normally we use 1 μl of labeled paint per slide (check with the provider for differences between batches). Dilute 1 μl of paint in 15 μl of formamide per slide to be hybridized, and proceed to **step 6**.
10. We use the same hybridization buffer for RNA FISH, DNA FISH and chromosome painting. For RNA FISH we add an RNase inhibitor (Vanadyl-ribonucleoside).
11. Put the slides in a staining trough, add enough H_2O to completely cover the slides, place the cover on top and put in a microwave at full power for 10 min.

12. At this point it is possible to store the ovary at −80 °C; place the ovary in a 1.5 ml tube, snap-freeze in dry ice and transfer to −80 °C. When ready you can proceed to **step 2** (ovaries can be safely stored for long periods). The RNA FISH signal will not be compromised by freezing. Use the all ovary to generate a batch of spreads, as meiotic progression is asynchronous and oocytes localized in different parts of the ovarian cortex are in slightly different phases of the process.
13. At this point the slides can be used immediately or stored in a box at −80 °C for later use (spreads can be safely stored at −80 °C for long periods).
14. An alternative way to add the probe is to place it on the coverslip and then invert the slide on top of the coverslip.
15. We put individual slides in a slide tray plate and the plate in a box containing paper towels soaked with 50% formamide in 2× SSC. Alternatively seal the slide with tip-top solution. In this case you have the choice of using a box in a 37 °C dry incubator, or of using a floating tray, covered with foil and placed in a 37 °C incubator bath. This last option is feasible with only a few slides.
16. In our experience DIG probes result in cleaner signal, therefore we prefer to use them for RNA FISH, or for single labeling experiments. The detection of biotin labeled probes is described in **steps 15–24** of Subheading 3.7.
17. Immunostaining is generally complementary to the RNA FISH protocol as it allows, amongst other aims, at least for identification and staging of the oocytes on the slide. We prefer performing it after the RNA FISH protocol to limit the possibility of RNA degradation. Check if the RNA FISH has worked by inspecting the slide under a fluorescence microscope. If the signal is fine transfer the slide to PBS and proceed with the immunostaining protocol specific to the antibodies to be used. The RNA FISH signal should survive the immunostaining treatment and the results from the two experiments can be imaged at the end. If RNA FISH has to be followed by DNA FISH or chromosome painting, do the immunostaining after the RNA FISH as indicated earlier. Record the images and then start the DNA FISH/chromosome paint protocol. Then record the new images. RNA and immunostaining signals may survive DNA FISH, but they become much weaker and a bit brittle because of the harsh treatment. It is therefore best to record the images before and after the DNA FISH. For this purpose it is necessary to use a fluorescence microscope equipped with a stage ensuring accuracy and precision of slide position and with motorized XY positioning. The XY coordinates of the cells imaged for RNA and antibody signals stored in a file are used after DNA FISH to retrieve the selected cells for reimaging.

18. This protocol generates better chicken oocyte spreads than the one described for the RNA FISH protocol and therefore it is preferred for immunohistochemistry with or without DNA FISH or chromosome painting, or, anyway, when the priority is a clear nuclear architecture.
19. We do not generally perform an RNase step before starting the chromosome painting/DNA FISH protocol on newly prepared slides, but if you have background problems, you can try troubleshooting by adding the RNase step at this point. After the rinse in 2× SSC, transfer the slides to a humidified chamber and add 100 µl of RNase solution per slide (100 µg/ml RNase A in 2× SSC). Incubate at 37 °C for 1 h and continue the protocol from **step 2**.
20. As mentioned in **Note 16**, in our experience DIG probes give cleaner signal in RNA FISH and DNA FISH, so we prefer to use DIG probes for single labeling experiments. This is not true for chromosome painting where both DIG or biotin probes produce equally good signals. When sequentially performing RNA and DNA FISH on the same slide we advise to use DIG probes for RNA FISH and biotinylated probes for DNA FISH because the detection of native RNA is trickier. When performing a simultaneous hybridization with two different probes, one labeled with biotin and the other with digoxigenin, always proceed by detecting first the biotinylated probe and then the DIG probe (this is valid for RNA FISH, DNA FISH, and chromosome painting).

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