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Fluorescence *in situ* Hybridization of Cryosectioned *Xenopus* Oocytes

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Abstract

Xenopus laevis oocytes provide an excellent resource for the study of RNA biology in vertebrate cells. Their large size provides advantages for both biochemical and imaging-based approaches. This strength has been applied to the study of RNA-protein interactions (Hake and Richter 1994; Gall et al. 1999; Yoon and Mowry 2004), polyadenylation (McGrew et al. 1989), translational control (Colegrove-Otero et al. 2005) and subcellular localization (Melton 1987; Mowry and Melton 1992) and transport of mRNA (Messitt et al. 2008; Gagnon et al. 2013). The ease with which distinct stages can be separated based on the morphology of the oocyte (Dumont 1972) allows for investigation of specific events in oogenesis in order to probe key developmental questions. However, the yolk-rich oocyte cytoplasm can present a barrier to high-resolution imaging of endogenous RNAs. Approaches to overcome these limitations include both electron microscopy and optical clearing of whole-mount preparations of oocytes and embryos (Kloc et al. 2005; Bilinski et al. 2010; Agricola and Cha 2016). Advances in fluorescence *in situ* hybridization (FISH), using probe sets of multiple fluorescently labeled oligonucleotides, has allowed for increased sensitivity and higher specificity (Raj et al. 2008). In addition, direct comparison of multiple RNA species in a single cell are possible through multiplexing of probe sets. This protocol combines the strengths of the multi-probe FISH approach with cryosectioning as a means of circumventing the need for clearing agents and increasing target accessibility in *Xenopus* oocytes.

MATERIALS

Reagents

- Ethyl Alcohol [190 Proof]
- Formamide (Fisher Scientific, BP228-100)
- MEMFA <R>
- MBSH Buffer <R>
- Nuclease-free H₂O (Thermo Fisher, AM9914G)
- O.C.T. Compound (Fisher Scientific, 4585)

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- Oocyte Culture Medium (OCM) <R>
- PBS <R>
- PBT <R>
- PBT-30S <R>
- Prolong Gold™ Antifade Mountant (Thermo Fisher Scientific, P36934)
- Richard-Allan Scientific™ Cytocool™ II Aerosol (Thermo Fisher Scientific, 8323)
- Silicone Adhesive Sealant (Loctite, 595)
- RNA FISH probe set for gene of interest (can be purchased from Biosearch Technologies, or synthesized as in Raj et al., 2008)
- Stellaris® RNA FISH Wash Buffer A (Biosearch Technologies, SMF-WA1-60)
- Stellaris® RNA FISH Wash Buffer B (Biosearch Technologies, SMF-WB1-20)
- Stellaris® RNA FISH Hybridization Buffer (Biosearch Technologies, SMF-HB1-10)
- Triton™ X-100 (Fisher Scientific, BP151)
- UltraPure™ BSA (Thermo Fisher Scientific, AM2616)
- RNase-ZAP™ (Ambion, AM9780)

Equipment

- 5 mL Eppendorf centrifuge tubes (Fisher Scientific, 14-282-300)
- Fluorescence (widefield or confocal) microscope.
- Cryostat (such as Leica CM3050 S Research Cryostat)
 - Glass Insert Anti-Roll Plate [70mm]
 - High Profile Disposable Microtome Blades 818 (Leica Biosystems, 14035838926)
 - Specimen Disc [30mm]
- Microscope Cover Glass [24×60-1]
- Microscope Slide Box
- Peel-A-Way® Disposable Embedding Molds [Truncated-T12], (Polysciences, 18986)
- RNase-free 5 ml Eppendorf tubes (Fisher Scientific, 14-282-300)
- Humidified Slide Chamber
- Superfrost Plus Gold Microscope Slides [25×75×1.0mm] (Thermo Fisher Scientific, 15-188-48)

- Sterile Cotton Tipped Applicators

METHODS

Following defolliculation and fine sorting of appropriately staged oocytes (Newman et al. 2017 for detailed protocol), oocytes should be incubated for a minimum of 1 hour in OCM to allow for recovery from defolliculation. Oocytes may be cultured overnight if desired.

A. Fixation and Freezing

1. Transfer a settled volume of 300–500 μ L of cultured oocytes in OCM to a 5 mL Eppendorf tube. Remove the OCM and wash oocytes with 5 mL of MBSH.
2. To fix, remove the MBSH and add 5 mL of MEMFA. Rotate the oocytes for 1 hour at room temperature.
3. Remove the MEMFA and replace with 5 mL of PBT. Wash oocytes for 20 minutes, rotating, at room temperature. Repeat PBT washes 2 times, for a total of 3 washes.
4. Following the final wash, allow oocytes to settle to the bottom of the Eppendorf tube. (*Note: some oocytes may adhere to the side of the Eppendorf and require gentle prodding with a pipette tip to be dislodged.*)
5. Remove 2.5 mL of PBT and replace with 2.5 mL of PBT-30S. Allow oocytes to equilibrate for 30 minutes at room temperature. (*Note: the oocytes will float to the top of the liquid volume upon addition of the PBT-30S and slowly sink to the bottom of the tube as they equilibrate.*)
6. Remove 5 mL of PBT/PBT-30S solution and replace with 5 mL of PBT-30S. Allow oocytes to equilibrate for 30 minutes at room temperature. Replace PBT-30S 3 times more for a total of 4 equilibrations.
7. Transfer the oocytes to a Peel-A-Way[®] Disposable Embedding Mold. Allow oocytes to settle to the base of embedding mold and carefully remove the PBT-30S from the embedding mold using a Pasteur pipette.
8. Gently add O.C.T. over the oocytes, filling the embedding mold to approximately the half waypoint. (*Note: avoid generating bubbles in the O.C.T, particularly near the sample.*) Allow oocytes to equilibrate in O.C.T. for 30 minutes at room temperature.
9. Snap freeze samples in a dry ice-ethanol bath by submerging the embedding mold to its midway point until the O.C.T. becomes uniformly solid. Store at -80°C .

B. Cryosectioning

1. Upon removal from -80°C all samples should be stored on dry ice prior to sectioning.

2. Place the oocytes (in embedding molds) into a precooled (-20°C) cryostat and equilibrate for approximately 30 minutes.
3. Remove sample from embedding mold and freeze it to a specimen disc using liquid O.C.T. and Cytocool II aerosol spray.
4. Cut 30–50 µM sections under a glass anti-roll plate.
5. Transfer each section to a room temperature microscope slide by touching the slide to the sample. (*Note: Each slide can hold 2–4 sections*)
6. Cure the sections to the slides by storing slides at 18–20°C overnight, shielded from light.

C. RNA Fluorescence *in situ* Hybridization (FISH)

1. Using a sterile cotton tipped applicator coated in silicone adhesive sealant, create a hydrophobic barrier around sectioned sample on the slide.
2. Rehydrate the sample by applying 1 mL of PBS to coat the sample within the hydrophobic well for 20 minutes at room temperature. (*Note: avoid application of liquid directly to the sectioned samples.*)
3. Remove the PBS and apply 1 mL of PBT to the sample. Incubate for 20 minutes at room temperature.
4. Remove the PBT and apply 1 mL of Stellaris® RNA FISH Wash Buffer A to the sample. Incubate for 5 minutes at room temperature.
5. Remove the Stellaris® RNA FISH Wash Buffer A and reapply 1 mL of Stellaris® RNA FISH Wash Buffer A. Incubate 5 minutes at room temperature.
6. Remove the Stellaris® RNA FISH Wash Buffer A and add 50–100 µL of Hybridization Buffer containing Stellaris RNA FISH probe at a concentration of 125–500 nM.
7. Carefully place a clean coverglass over the Hybridization Buffer to completely cover and evenly distribute the Hybridization Buffer across the sample.
8. Place the slide in a humidified slide chamber and incubate in the dark for 16–20 hours at 37°C.
9. Immerse the slide in Stellaris® RNA FISH Wash Buffer A and use forceps to gently remove the coverglass.
10. Remove the slide from the Stellaris® RNA FISH Wash Buffer A and remove excess buffer surrounding the sections.
11. Apply 1 mL of Stellaris® RNA FISH Wash Buffer A to the slide and incubate in the dark for 30 minutes at 37°C.
12. Remove Stellaris® RNA FISH Wash Buffer A and apply 1 mL of Stellaris® RNA FISH Wash Buffer B. Incubate in the dark for 5 minutes at room temperature.

13. Remove Stellaris® RNA FISH Wash Buffer B and reapply 1 mL of Stellaris® RNA FISH Wash Buffer B. Incubate 5 minutes at room temperature, shielded from light.
14. Remove Stellaris® RNA FISH Wash Buffer B and add approximately 50–100 µL of Prolong Gold™ Antifade Mountant onto the sample sections and cover with a clean coverslip.
15. Seal corners using nail polish. Cure the samples at room temperature for 24 hours shielded from light.
16. Seal the coverglass perimeter with nail polish and allow to dry shielded from light.

Samples are now suitable for imaging using a fluorescence microscope. For short-term storage (up to a week), slides are stable at 4°C.

TROUBLESHOOTING

Problem

Poor retention of oocytes on slides during RNA FISH treatment (Steps C2-19)

Solution

Increasing the density of oocytes on the slide will significantly improve sample retention during processing. Ensuring that oocytes are uniformly settled along the base of the embedding mold prior to snap freezing generates an optimal cutting surface to produce more durable sections.

RECIPES

MBSH (10X)

Store as a sterilized 10X stock solution and prepare fresh 1X MBSH prior to use.

880 mM NaCl
 10 mM KCl
 24 mM NaHCO₃
 8.2 mM MgSO₄·7H₂O
 3.3 mM Ca(NO₃)₂·4H₂O
 4.1 mM CaCl₂·6H₂O
 100 mM HEPES (pH 7.6)

MEMFA

Prepare fresh, and protect from light.

1 ml 10× MEM (1 M MOPS, pH 7.4, 20 mM EGTA, 10 mM MgSO₄)

1 ml microfiltered 37% formaldehyde (Electron Microscopy Science, 15686)

8 ml deionized H₂O

Oocyte Culture Media

50% Leibovitz's L-15 Medium (Thermo Fisher Scientific, 11415064)

15 mM HEPES, pH 7.6

1 mg/ml Insulin

Sterilize through a 0.22 µm filter—*OCM without antibiotics can be stored at 4°C for up to two months.*

Add antibiotics to OCM just prior to culture. Do not store. Do not reuse.

50 U/ml Nystatin (Sigma-Aldrich, N1638)

100 U/ml Penicillin/Streptomycin (Life Technologies, 15070063)

0.1 mg/ml Gentamicin (Thermo Fisher Scientific, 15750060)

PBS

137 mM NaCl

2.7 mM KCl

10 mM Na₂HPO₄

1.8 mM KH₂PO₄

PBT

1× PBS supplemented with

0.2mg/mL BSA (Sigma-Aldrich, A2153)

0.1% (v/v) Triton X-100 in 1×PBS.

Mix thoroughly and sterilize through 0.22 µm filter. Store at 4°C.

PBT-30S

1× PBT supplemented with

30% (w/v) Sucrose

Mix thoroughly. Store at 4°C

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Figure 1.

(A) A workflow diagram depicts how oocyte samples cryosectioned onto a microscope slide (Method B) are outlined with silicone adhesive sealant using a cotton tip applicator (Method C1), which creates a hydrophobic well over the sample in which FISH treatments (Methods C2-13) are conducted. Detection and localization of endogenous Vg1 (Weeks and Melton 1987), Xpat (Hudson and Woodland 1998) and GAPDH mRNA in a Stage I-II oocytes were performed using Stellaris gene specific probe sets designed and provided by LGC BioSearch Technologies. The Vg1 (Quasar® 570), Xpat (Quasar® 570) and GAPDH (Quasar® 670) probe sets were applied at a concentration of 250 μ M and imaged using a Zeiss LSM 800 Confocal Laser Scanning Microscope with a 20 $\times$ objective. (B) A stage I oocyte is shown with ubiquitously distributed GAPDH mRNA (green) and Xpat mRNA (red), which is localized to the Balbiani body. (C) A stage II oocyte is shown with Vg1 mRNA (red), which is enriched in the vegetal hemisphere. Oocytes are oriented with the vegetal pole at the bottom, and scale bars = 50 μ M.