

RNA Imaging with MERFISH - Sample Preparation and Staining

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Abstract

The preparation and staining of samples for MERFISH follows closely the typical protocols used for smFISH (Raj et al., 2008). However, there are a few places in which we have modified these protocols to optimize MERFISH staining. Again, RNase contamination can destroy samples, so care should be taken to work in an RNase-free environment.

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Guidelines

The protocol workflow is as follows:

1. Fixation and Permeabilization of Cells (Steps 1-5)

The first step in most FISH protocols is to fix the cells and then to permeabilize the membrane using either an overnight incubation in 70% ethanol or a brief exposure to surfactant. We prefer the latter approach since this protocol requires less time; however, we have had reasonable performance with both approaches.

2. Hybridization of Encoding Probes (Steps 5-23)

This protocol will require the following reagents and buffers:

1. Fixation buffer (4% PFA in 1XPBS)

- 1 mL 32% paraformaldehyde (PFA; Electron Microscopy Sciences; 15714)
- 0.8 mL 10X Phosphate Buffered Saline (PBS; Ambion; AM9625)
- 6.2 mL nuclease-free water
- Store at -20°C in single use aliquots

2. Permeabilization buffer (0.5% v/v Triton X-100 in 1XPBS)

- 5 mL 10X PBS
- 45 mL nuclease-free water
- 250 μL Triton X-100 (Sigma; T8787)
- Store at room temperature

3. 2X SSC buffer

- 5 mL 10X PBS
- 45 mL nuclease-free water
- 250 μL Triton X-100 (Sigma; T8787)
- Store at room temperature

4. 1X PBS buffer

- 5 mL 10X PBS
- 45 mL nuclease-free water
- Store at room temperature

5. 20% w/v Dextran sulfate

- In an RNase-free bottle (such as an empty nuclease-free water bottle), mix the following
- 100 g dextran sulfate (Fisher Scientific; bp1585100)
- 300 mL nuclease-free water
- Stir with a stir bar cleaned with D/RNaseFree while heating gently on a hot plate (37°C) until dissolved
- Add nuclease-free water to produce a 500 mL total volume
- Store at room temperature

6. Encoding probe hybridization buffer

- 300 μL 100% deionized formamide (Ambion; AM9342)
- 500 μL 20% w/v dextran sulfate (above)
- 100 μL 20X SSC
- 90 μL nuclease-free water

- 1 mg yeast tRNA (Life Technologies; 15401-011)
- 10 μ L 200 mM Vandyl Ribonucleoside Complex (VRC; New England Biolabs; S1402S)
- Store at -20°C

7. Encoding probe wash buffer

- 15 mL 100% deionized formamide
- 5 mL 20X SSC
- 29.5 mL nuclease-free water
- 0.5 mL 200 mM VRC
- Store at 4°C in the dark and use within a few days

8. 40-mm diameter, No. 1.5 coverslips (Bioptechs; 40-1313-0319)

9. 60-mm diameter, Cell culture petri dish (Corning; 353802)

10. 1" x 3" microscope slides

11. Parafilm (VWR; 52858-076)

12. Orange FluoSphere carboxylate-modified beads, 0.1- μ m-diameter (ThermoScientific; F-8800)

This protocol requires the following equipment:

1. Equipment for cell culture
2. 37°C incubator
3. 47°C incubator
4. Laboratory rocker

Materials

✓ Please see Before starting section for required materials by Contributed by users

Protocol

Fixation and Permeabilization of Cells

Step 1.

Culture cells on a suitable coverglass. The culture substrate for cells should be the same coverglass that will be used for imaging. We utilize 40-mm diameter No. 1.5 coverslips from Biopetechs since these coverslips fit within the flow chamber that we use for [imaging](#). We typically culture cells in sterile, 60-mm-diameter petri dishes with one coverslip placed in the bottom of each dish.

Fixation and Permeabilization of Cells

Step 2.

Fix cells. Allow the fixation buffer described in the Before Starting section to warm to room temperature. Aspirate culture medium from cells then gently decant 2-3 mL of fixation buffer into the petri dish that holds the coverslip. Cover and rock gently for 15 minutes at room temperature.

Fixation and Permeabilization of Cells

Step 3.

Aspirate the fixation buffer and gently decant 2-3 mL of 1X PBS into the petri dish to wash the cells. Immediately aspirate this buffer. Repeat this 1X PBS wash for a total of three times.

📄 [AMOUNT](#)

3 ml Additional info: 1X PBS

Fixation and Permeabilization of Cells

Step 4.

Permeabilize the cells. Aspirate any residual 1X PBS from the petri dish and decant 2-3 mL permeabilization buffer into the petri dish. Incubate the cells at room temperature with gentle rocking for 2 minutes.

📄 [AMOUNT](#)

3 ml Additional info: Permeabilization buffer

Fixation and Permeabilization of Cells

Step 5.

Aspirate out the permeabilization buffer and wash the cells three times with 1X PBS as described in Step 3.

Hybridization of Encoding Probes

Step 6.

Exchange buffer on cells. Aspirate any residual 1X PBS, and gently decant 2-3 mL of room temperature encoding probe wash buffer into each petri dish. Rock the sample at room temperature for 5 minutes.

📄 [AMOUNT](#)

3 ml Additional info: Probe wash buffer

📌 NOTES

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This step insures that any residual buffer left on the coverslip will not significantly dilute the formamide in the hybridization buffer in the next step.

Hybridization of Encoding Probes

Step 7.

Add hybridization buffer with probes. Place a layer of fresh parafilm on a 1" x 3" microscope slide. Dilute the encoding probes made in the [Probe Construction](#) protocol into Encoding Probe Hybridization Buffer to the desired concentration, typically somewhere between 10 and 200 μ M depending on the number of unique encoding probes in the probe set.

Hybridization of Encoding Probes

Step 8.

Add 30 μ L of this probe solution to the parafilm surface.

📌 AMOUNT

30 μ L Additional info: Probe solution

Hybridization of Encoding Probes

Step 9.

Aspirate any residual encoding probe wash buffer from the coverslip and gently place the coverslip, cell-side-down, onto the droplet of encoding probes. Press gently to create a thin layer of encoding probe.

📌 NOTES

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The probe concentration needs to be optimized for each probe set: Higher probe concentrations produce brighter spots but a higher level of non-specifically bound probe background. We recommend titrating the concentration of probe across a ~40-fold concentration range (5 – 200 μ M) to identify the concentration that produces the clearest signal relative to the background. We find that the optimal probe concentration only needs to be established once per probe set.

Hybridization of Encoding Probes

Step 10.

Incubation. Create a humidity chamber by pouring nuclease-free water into the base of an old pipette tip box.

Hybridization of Encoding Probes

Step 11.

Place the sample within this box and seal with parafilm.

NOTES

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The sample will be sitting on the upper plastic level and should not come into contact with the water reservoir below.

Hybridization of Encoding Probes

Step 12.

Place this box into a 37 °C incubation chamber and incubate for at least 12 hours.

TEMPERATURE

37 °C Additional info: Incubation chamber

NOTES

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In some cases, we find that longer incubations (~36 hours) increase the quality of staining. Again, we recommend varying the incubation time for each probe set to identify the optimal incubation time.

Hybridization of Encoding Probes

Step 13.

Wash away residual encoding probes. Preheat 6 mL of encoding probe wash buffer per sample to 47 °C in a water bath.

AMOUNT

6 ml Additional info: Encoding probe wash buffer per sample

TEMPERATURE

47 °C Additional info: Water bath

Hybridization of Encoding Probes

Step 14.

Slowly peel the coverslip off of the microscope slide taking care not to crack the coverslip. If it appears to be stuck, immerse the assembly in a layer of encoding probe wash buffer for 5 minutes to loosen.

Hybridization of Encoding Probes

Step 15.

Place the coverslip, cell-sideup, in a fresh 60-mm-diameter petri dish and add 3 mL of preheated encoding probe wash buffer.

AMOUNT

3 ml Additional info: Preheated encoding probe wash buffer

Hybridization of Encoding Probes

Step 16.

Place the petri dish in a 47-°C incubator for 30 minutes.

TEMPERATURE

47 °C Additional info: Incubator

Hybridization of Encoding Probes

Step 17.

Aspirate the encoding probe wash buffer, add 3 mL of fresh encoding probe wash buffer, and repeat the 30-minute, 47-°C incubation.

AMOUNT

3 ml Additional info: Encoding probe wash buffer

TEMPERATURE

47 °C Additional info: Incubator

Hybridization of Encoding Probes

Step 18.

Aspirate the wash buffer and wash the sample in 2X SSC by adding 3 mL of 2X SSC and then immediately aspirating this buffer. Repeat this SSC wash for a total of three times.

AMOUNT

9 ml Additional info: 2X SSC

Hybridization of Encoding Probes

Step 19.

Addition of fiducial beads. Prepare a 100X solution of fluorescent beads by adding 10 µL of 0.1-nm-diameter Orange Fluospheres beads to 10 mL 2X SSC. Vortex to mix. This stock solution can be stored at 4 °C in the dark for several months. Further, dilute this stock solution 1 to 100 in 2X SSC to create 3 mL of bead solution for each sample.

AMOUNT

10 µl Additional info: 0.1-nm-diameter Orange Fluospheres beads

AMOUNT

10 ml Additional info: 2X SSC

Hybridization of Encoding Probes

Step 20.

Aspirate the 2X SSC from the samples and add the bead solution to each petri dish. Gently rock at room temperature for 15 minutes.

NOTES

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We choose orange-colored beads (561-nm excitation) because we prefer to use the red imaging channel (641-nm excitation) for our smFISH images; however, the color of these beads could be changed to suit different imaging demands. We aim to have ~20 beads per field of view; thus, if different beads are used, the concentration should be adjusted to produce a similar final density of beads on the sample.

Hybridization of Encoding Probes

Step 21.

Post-fixation. To fix the fiducial beads in place and to prevent subtle changes in the shape of the cell induced by the repeated washing that will occur in the MERFISH Imaging protocol, we perform an additional fixation of the samples. Aspirate the bead solution and add 3 mL of fixation buffer prewarmed to room temperature to each petri dish.

AMOUNT

3 ml Additional info: Fixation buffer

Hybridization of Encoding Probes

Step 22.

Gently rock the sample at room temperature for 10 minutes.

Hybridization of Encoding Probes

Step 23.

Aspirate the fixation buffer, and wash the sample three times with 2X SSC.

NOTES

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If the sample was prepared in an RNase-free environment, it can now be stored in 2XSSC at 4°C for several days. We do not recommend prolonged storage.