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Single molecule FISH

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1 Works for me dx.doi.org/10.17504/protocols.io.xb2fiqe

CZI Spatial Transcriptomics Protocol Repository

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ABSTRACT

This protocol describes multiround hybrization of directly-conjugated FISH probes for single molecule RNA detection. Thin tissue sections (10-µm) are placed onto silanized coverslips (24x50) that fit onto an ASI imaging chamber. A SecureSeal chamber is placed around the sections, which act as a reaction chamber and imaging chamber.

MATERIALS

NAME	CATALOG #	VENDOR
4% PFA		
Coverslip #1.5H 24x50 (Thorlabs)	CG15CH	Thorlabs
3-aminopropyltriethoxysilane (APES)	A3648	Sigma
ASI Coverglass Imaging Chamber	I-3078-2450	ASI Imaging
SecureSeal Chambers 13mm x 0.8mm	621502	Grace Bio-Labs

Coverslip Preparation

1 Clean coverslips (Thorlabs #CG15KH) with lens paper and 70% ethanol. With minimal handling of the coverslips (touch edges with clean gloves is ok), load them into a coverslip rack compatible with a plasma cleaning oven. We use a quartz coverslip rack. Placing coverslips in a clean glass container is ok.

2 Place coverslips into a plasma oven. Turn on oven. Form vacuum. Turn on plasma and let clean for 30 mins.

Pipette 100 µl of 3-aminopropyltriethoxysilane (APES, Sigma #A3648) onto a crumpled KimWipe and place next to coverslips.

3 Transfer coverslips to vacuum dessicator. Pipette 100 µl of 3-aminopropyltriethoxysilane (APES, Sigma #A3648) onto a crumpled KimWipe and place next to coverslips. Form vacuum and let sit for 10 min. To remove free silanes, wash coverslips by moving up and down 5 times in 100% methanol, then in fresh methanol before allowing to dry.

4 Store cleaned and silanized coverslips in a cool, dry, place with minimal dust.

We have used them after 2 months of storage without issues. It's possible they could last longer.

Tissue Sectioning

5 Adhere silanized coverslips to glass slides to help with picking up sections. Place 10 µl of nuclease-free water onto a clean slide and place a silanized coverslip on top. The water will hold the coverslip onto the slide during sectioning.

6 Place the frozen OCT block with embedded brain into a -20°C cryostat for 30 minutes to equilibrate to temperature.

7 Using a cryostat, make 10-µm sections and pick up on pre-chilled silanized coverslips. Let sections dry in the cryostat chamber for 30 minutes. Proceed immediately to Fixation and Permeabiliization.

Tissue Fixation and Permeabilization

8 Reagent Prep:
- 4% PFA in PBS (make fresh) and pre-chill at 4°C
- 100% methanol pre-chill at -20°C
- 1X PBS made with nuclease-free water

9 Fix the tissue by placing the coverslips (attached to slides) into a slide rack and immerse in pre-chilled 4% PFA in PBS for 15 minutes at 4°C.

The coverslips will detach from the slides at this point. Let fix for 15 mins and then remove the coverslips (without slides) and into a coverslip rack for washing.

10 Wash 3x 5 mins with PBS.

11 Permeabilize with 100% methanol for 10 mins at -20°C.

12 Remove coverslips from the methanol and let dry completely at room temperature.

13 Box the coverslips into experimental sets. For example, 2-6 coverslips per box. Place each box into a zip lock bag. Seal tight. Store at -80°C.

When ready to use, remove each box and use all coverslips. Do not place back into freezer once the zip lock bag and box is opened. We have used stored tissue slices up to three weeks without noticeable issues.

Tissue Preparation

14 Reagent Prep:
- 8% SDS in PBS (vacuum filter, store at room temperature)
- 2X SSC (vacuum filter)
- SecureSeal Chambers (Grace Bio-Labs #621502)

15 Remove coverslips from -80°C and place on a glass slide for support.

16 Trim SecureSeal chambers so they fit within the open footprint of the ASI Imaging Chamber or equivalent, and place it around each section. It is important that all handling and room temperature steps from this point forward be performed in a clean PCR hood maintained as an RNase-free area. Samples should be covered, either in a humidified chamber or with port seal stickers, before transfer to the 37°C oven, 4°C fridge or microscope for imaging.

17 Pipette 2X SSC into the chamber slowly, making sure to push out the air and not introduce air bubbles. Let tissue section equilibrate to room temperature for 1 min.

Take note of how much volume is needed to fill the chamber. It is 100 µl for the chambers specified in this protocol. For reagents that need to be at a specific concentration, we typically push 200 µl through the chamber to make sure the last 100 µl is at the desired concentration.

18 Pipette 200 µl of 8% SDS into the chamber. Let incubate for 10 mins at room temperature.

19 Holding the coverslip sideways over a beaker, pipette 500 µl of 2X SSC through the chamber and let the buffer flow out the other port and fall into the beaker. Repeat 4 more times or until the output buffer is no longer soapy. All solutions should be added as slowly as possible.

Hybridization

20 Reagent Prep:

- Probes (take out to thaw)
- 2X SSC
- DAPI (5 µg/ml in 2X SSC)
- Hybridization Buffer: 10% formamide in 2X SSC with tRNA, RVC, and BSA (make aliquots and store at -20°C for up to three months)
- Probe Wash Buffer: 20% formamide in 2X SSC

Reagent	Amount	Final Concentration	Notes
Dextran sulfate	1 g	100 mg/ml	
Nuclease-free water	7.3 ml		Dissolve dextran sulfate in water, then add other reagents.
20X SSC	1 ml	2X	
De-ionized formamide	1 ml	10%	
tRNA	500 µl	1 mg/ml	Sigma #1019541001. Make 20 mg/ml stock.
Ribonucleoside Vanadyl Complex	100 µl	2 mM	NEB #S14025
BSA	40 µl	200 µg/ml	Ambion #AM2616. Stock is 50 mg/ml.
Final Volume	10 ml		

Hybridization Buffer

- Imaging Buffer (store at 4°C for one month)

Reagent	Amount	Final Concentration	Notes
Glucose	0.4 g	8 mg/ml	
Nuclease-free water	48.5 ml		Dissolve glucose in water, then add other reagents.
1 M Tris-HCl, pH 8.0	1 ml	20 mM	
5 M NaCl	500 ul	50 mM	
Final Volume	50 ml		

Imaging Buffer without Enzymes

21 Equilbrate tissue section in Hybridization Buffer (without probes) by pipetting 100 µl of the buffer into the SecureSeal chamber. Incubate for 5 mins.

22 Make probe mix while tissue sections are equilibrating in HB. Dilute probes in HB to final concentration of 2 ng/µl.

23 Pipette the probe mix into the SecureSeal chamber. Cover ports with the port sealing stickers included with the SecureSeal pack. Place into a humified chamber and incubate for 2 hours at 37°C.

Humidified chamber can be an empty pipette tip box. Place several wet paper towels at the bottom of the box. Replace the tip rack and place the coverslips on the rack. Cover with a lid and seal with Parafilm.

24 Wash with Probe Wash Buffer containing 5 µg/ml DAPI for 10 mins at 37°C. Push 500 µl through while holding the coverslip over a beaker.

25 Wash with Probe Wash Buffer 2 x 10 min at 37°C. Push 500 µl through while holding the coverslip over a beaker.

26 Hold the coverslip over a beaker and pipette 500 µl of 2X SSC through the chamber. Repeat once.

27 Add enzymes to the Imaging Buffer (see table below). Pipette Imaging Buffer (with enzymes) to the SecureSeal chamber. Proceed to imaging.

Reagent	Amount	Final Concentration	Notes
Imaging Buffer without Enzymes	1 ml		
250 U/ml pyranose oxidase	12 µl	2.4 U/ml	Sigma #P4234-250U
8 mg/ml catalase	6.25 µl	50 µg/ml	Abcam #ab219092
200 mM trolox in ethanol	2.5 µl	500 µM	

Imaging Buffer with Enzymes

Add enzymes to Imaging Buffer and use immediately. Do not store.

Imaging

28 Use appropriate Imaging Protocol.

Probe Stripping

29 Reagent Prep:
- 2X SSC
- 65% formamide in 2X SSC (vacuum or syringe filter)

30 Replace Imaging Buffer in the SecureSeal chamber with 2X SSC.

Optional stopping point: Store at 4°C overnight.

31 Add 65% formamide in 2X SSC to the SecureSeal chamber for 3x 10 mins at 37°C.

We performed this step at 37°C for this protocol. However, 30°C has also been used with success.

32 Wash with 2X SSC 3x 5 mins at room temperature by pipetting 500 µl through the chamber each time.

Optional stopping point: Store at 4°C overnight.

33 Proceed to hybridization. See section above.

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