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Single Molecule RNA FISH (smFISH) in Whole-Mount Mouse Embryonic Organs

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Abstract

Single molecule RNA fluorescence in situ hybridization (smFISH) has become the standard tool for high spatial resolution analysis of gene expression in the context of tissue organization. This unit describes protocols to perform smFISH on whole-mount mouse embryonic organs, where tissue organization can be compared to RNA expression by co-immunostaining of known protein markers. An enzymatic labeling strategy is also introduced to produce low-cost smFISH probes. Important considerations and practical guidelines for imaging smFISH samples using fluorescence confocal microscopy are described. Finally, a suite of custom-written ImageJ macros is included with detailed instructions to enable semi-automated smFISH image analysis of both 2D and 3D images.

Keywords

smFISH; enzymatic probe labeling; co-immunostaining; ImageJ macro quantification

INTRODUCTION

It is often important to determine the location of gene transcripts in developmental biology studies. Traditionally, this is achieved by RNA in situ hybridization (ISH), which relies on the hybridization of hapten-labeled DNA or RNA probes to the target RNA (Tautz and Pfeifle, 1989). The hapten labels, such as digoxigenin or biotin, are then recognized by antibodies or streptavidin linked to enzymes that catalyze chromogenic or fluorogenic reactions to produce detectable signals (Rosen and Beddington, 1993; Raap et al., 1995). The ISH techniques using hapten-labeled probes have fueled many discoveries, but they suffer from either low sensitivity or low selectivity depending on the signal generation and amplification strategies. Moreover, they all have limited spatial resolution due to diffusion of the chromogenic or fluorogenic molecules used for detection.

Single molecule resolution of RNA was first achieved as a variation of fluorescence in situ hybridization (FISH) (Femino et al., 1998). In this method, a small number (5) of multiply labeled short oligonucleotide probes (about 50 nucleotides long, each with 5 fluorescent molecules) were used to target the same RNA to generate a detectable diffraction-limited fluorescent spot. Subsequently, an improved design using a much larger number (30–50) of singly labeled shorter probes (18–22 nucleotides) has greatly simplified the probe labeling procedure, and has also improved its selectivity and sensitivity (Raj et al., 2008; Figure 1A).

This method has since been widely used and referred to as smFISH (single molecule FISH). For example, our lab has successfully used smFISH to examine gene expression in whole-mount mouse embryonic organs (Daley et al., 2017; Figure 1B–D). In the era of next generation sequencing, smFISH and its various derived methods provide important complementary approaches to examine gene expression in the context of tissue organization.

This unit includes a set of protocols for applying smFISH in whole-mount mouse embryonic organs. *Basic Protocol 1* describes in detail the steps of sample preparation, smFISH staining and co-immunostaining. *Alternate Protocol 1* describes a simplified co-immunostaining protocol for high-abundance protein markers. *Support Protocol 1* describes an enzymatic labeling procedure to produce low-cost smFISH probes from standard desalting-purified DNA oligonucleotides (Gaspar et al., 2017). *Basic Protocol 2* describes important considerations and procedures to image smFISH samples using fluorescence confocal microscopy. Finally, *Basic Protocol 3* provides detailed guidelines to perform semi-automated smFISH image analysis using a suite of custom-written ImageJ macros.

BASIC PROTOCOL 1

Single Molecule FISH and Co-immunostaining of Whole-Mount Mouse Embryonic Organs

Introductory paragraph—The purpose of this protocol is to examine gene expression at high spatial resolution by smFISH in whole-mount mouse embryonic organs, such as salivary glands, lungs and kidneys. Optionally, these patterns of gene expression can be placed into the context of tissue organization by co-immunostaining of known protein markers. Mouse embryonic organs at their early developmental stages are usually only a few hundred microns in diameter and 100–200 microns in thickness, which makes them difficult to section while still retaining tissue integrity. On the other hand, their relatively small size makes it practical to perform smFISH in whole-mount samples, which maximally preserve tissue integrity.

The general steps of this protocol include sample isolation, fixation, dehydration, rehydration, permeabilization, probe hybridization, nuclear co-staining, and optional co-immunostaining of protein markers. Because the complete processing of samples takes 2–4 days (depending on whether co-immunostaining is performed), it is important to become familiar with the entire protocol before starting. A schematic of the complete smFISH experiment workflow is provided to help with experimental planning (Figure 2A).

Materials

Reagents and Solutions:

- 25 μ M Stellaris smFISH Probes from LGC Biosearch Technologies (see *Step 1*)
- Ultrapure Water (e.g., Quality Biological, 351-029-131)
- Organ Culture Media (see recipe)
- Fixative Solution (see recipe)
- PBSTx (see recipe)

PBS-SDS (see recipe)

Wash Solution (see recipe)

Hybridization Solution (see recipe)

DEPC-treated Water (e.g., Quality Biological, 351-068-131)

Methanol

DAPI Stock Solution (see recipe)

2× SSC (see recipe)

Donkey Serum (Jackson ImmunoResearch, 017-000-121)

Rat Anti-E-cadherin Antibody (Thermo Fisher, 13–1900)

Goat Anti-collagen IV Antibody (MilliporeSigma, AB769)

Alexa Fluor 647 (AF647) Conjugated Donkey Anti-Rat Secondary Antibody (Jackson ImmunoResearch, 712-605-153)

Cy2 Conjugated Donkey Anti-Goat Secondary Antibody (Jackson ImmunoResearch, 705-225-147)

75 × 25 mm Glass Slides (e.g., VWR 48311–703)

Imaging Spacers (Grace Bio-labs, 654004)

ProLong Diamond Antifade Mountant (Thermo Fisher, P36961)

Fluoro-Gel with TES Buffer (Electron Microscopy Sciences, 17985–31)

22 × 22 mm #1.5 Thickness Coverslips (e.g., Fisher Scientific, 12-518-213)

Cardboard Slide Tray (Fisher Scientific, 12-587-10)

Sample Material—Timed pregnant mouse at desired gestational stage; for demonstration of the protocol, a pregnant ICR (CD-1) outbred mouse at E13 stage from Envigo was used.

Other Materials and Equipment:

Nuclease-free 1.6 mL Polypropylene Tubes (e.g., VWR, 4445.S.X)

Nuclease-free 2 mL Polypropylene Tubes (e.g., Eppendorf, 022600044)

Nuclease-free 15 mL and 50 mL Polypropylene Conical Tubes (e.g., Corning, 430766 and 430291)

20 mL Glass Vials (e.g., Fisher Scientific, 03-340-25N)

Sample Baskets (e.g., Intavis, 12.440)

Dissection Microscope

Forceps (e.g., Fine Science Tools; 11254-20)

Polycarbonate Filter (Sigma-Aldrich, WHA110405 or WHA110406)

50 mm MatTek Dish with 14 mm Opening (MatTek, P50G-1.5–14-F)

Humidified 37°C Incubator with 5% CO₂ for Organ Culture

Laboratory Rocker

Table-top Centrifuge

24-well Plate (e.g., Fisher Scientific, 09-761-146)

35 mm Dish (e.g., Fisher Scientific, 08-772A)

Aluminum Foil

Humidified 37°C Incubator (without CO₂) for Hybridization Incubation

Protocol steps—*Step annotations*

1. Design and order smFISH probes for your RNAs of interest using the Stellaris probe designer (free with registration) at: <https://www.biosearchtech.com/support/tools/design-software/stellaris-probe-designer>. LGC Biosearch Technologies has a helpful article that discusses important considerations for smFISH probe design: <http://blog.biosearchtech.com/considerations-for-optimizing-stellaris-rna-fish-probe-design>.

In general, using more probes for the same target RNA generates better signal-to-noise ratio. At least 25 probes that are 18–22 nucleotides in length should be used to ensure robust detection. There should be a gap of at least 2 nucleotides between the sequences of adjacent probes to avoid interference during hybridization. The GC content of each probe should ideally be around 50%.

Although it is possible to order probes labeled with fluorescent dyes ranging from green to far-red wavelengths, I have found orange dyes such as tetramethylrhodamine (TMR) and Quasar 570 are the brightest and most resistant to photo bleaching among currently available choices. Green dye is usually not good for tissue samples due to high background at this wavelength – I have not attempted to use green dye-labeled probes for smFISH in mouse embryonic organs. The far-red dye Quasar 670 can be used for multiplexing with TMR or Quasar 570, but it is dimmer and more prone to photo bleaching. Therefore, use the brighter orange dye (TMR or Quasar 570) for the RNA for which you have fewer probes when multiplexing.

The custom-designed probe sets from LGC Biosearch Technologies come as a dry pellet typically at a scale of 5 nmol. Dissolve the pellet in 200 µL of Nuclease-free water to obtain a probe stock solution at 25 µM. Pipet the stock into 20 µL aliquots, and store them frozen at –20°C or –80°C. The probes can be thawed at least 5 times without affecting performance. You can also store an aliquot at 4°C for usage within a month, but probes do deteriorate when stored for longer at 4°C.

2. (Day 1) Before mouse dissection, prepare 1 mL of Organ Culture Media, 1 mL of Fixative Solution, 30 mL of PBSTx, 2 mL of PBS-SDS, 2 mL of Wash

Solution and 1 mL of Hybridization Solution in nuclease-free tubes according to the recipes listed under *Reagents and Solutions*.

If you begin dissection late in the day, and choose to do overnight sample fixation (see Step 6), make the Wash Solution and Hybridization Solution on the next day.

The volumes indicated here are sufficient for collecting up to 40 small-size embryonic organs (such as E13 salivary glands) cultured on 5 polycarbonate filters, and for staining 2 groups of samples. Adjust the volumes based on your sample number.

Since PBSTx and PBS-SDS can be stored at room temperature (RT) for at least 3 months, you can make them in any convenient volumes (e.g., 50 mL).

Hybridization Solution can be stored frozen at -20°C and thawed at least 10 times without affecting performance, so you can make 10 mL and thaw the entire volume in a 37°C water bath before use. Organ Culture Media, Fixative Solution and Wash Solution should be made fresh on the day of usage.

3. Clean two 20 mL glass vials and two sample baskets by rinsing them with clean water (such as Milli-Q purified water, double-distilled water or ultrapure water) 3 times, and then once with DEPC-treated water. After cleaning, store the sample baskets in DEPC-treated water in a 50 mL conical tube until usage.

You will need 2 glass vials for sample processing and storage for each experimental group of samples depending on their genotype, treatment and developmental stage. For each experimental group, you will need 1 sample basket for each RNA you want to examine by smFISH, unless you are multiplexing RNAs using different colors.

For example, if you want to examine 3 RNAs in mouse embryonic salivary glands of 2 genotypes, you will need 4 glass vials (for storage and processing) and 6 sample baskets (for staining). The salivary glands can be easily distinguished from lungs and kidneys by morphology, so they can be conveniently pooled together for staining. However, early-stage lungs and kidneys can look quite similar when imaging a small field of view under the microscope, so it is recommended to separate lungs and kidneys into different experimental groups.

4. Isolate salivary glands, lungs or kidneys by dissection from mouse embryos at desired stages using forceps under a dissection scope following organ-specific procedures described previously (*Unit 19.8; Sakai and Onodera, 2008*).
5. After dissection, culture 4–8 isolated organs on a polycarbonate filter (13 mm diameter) floating on 200 μL of Organ Culture Media in the middle opening (14 mm diameter) of a 50-mm diameter MatTek dish. Incubate in a humidified 37°C incubator with 5% CO_2 for 1 hour to let the organs adhere to the filter.

Note: Brief culturing on the filter makes organs flatten onto the filter. This flattening makes the organs much easier to image using a microscope.

1-hour culturing time is sufficient for E12–14 stage salivary glands, E12–13 stage lungs and kidneys to adhere to the filter, but maybe insufficient for organs at earlier or later stages. The culture time can be extended to make them adhere better. Alternatively, when the organs come off the filter during fixation, transfer them into sample baskets placed in the wells of a 24-well plate for processing.

6. Fix the samples by replacing the Organ Culture Media under the filter with 200 μ L of Fixative Solution. Keep the Fixative Solution under the filter to allow organs to firmly attach to the filter during fixation. Incubate for 1 hour at RT with gentle rocking on a laboratory rocker.

Note: If you begin the dissection late in the day, you can fix the samples overnight at 4°C. For overnight fixation, place the organ culture dish inside a humidified chamber.

Fixation time may need to be adjusted for late-stage organs that are much larger in size. However, those organs of much larger size are probably more suitable for sectioning instead of whole-mount processing.

7. During fixation, mix 2.4 mL, 4 mL and 5.6 mL of methanol with 5.6 mL, 4 mL and 2.4 mL of PBSTx in 15 mL tubes to obtain 8 mL of 30%, 50% and 70% methanol in PBSTx. Place the methanol concentration series on ice, and pre-cool a pre-cleaned 20 mL glass vial filled up with 100% methanol at –20°C.
8. Use a pair of forceps to transfer all filters with fixed samples of the same experimental group to another pre-cleaned 20 mL glass vial filled with 4 mL of PBSTx. Make sure all filters are immersed in the solution. Rinse for 5 min at RT with gentle rocking on a laboratory rocker.

Transferring all filters into a glass vial allows batch processing of many samples. The vial can also be used for long-term sample storage after dehydration. It is also fine to use a different type of container, such as a 35-mm dish or a 24-well plate.

9. Dehydrate samples in each glass vial using 4 mL each of an increasing methanol concentration series (30%, 50% and 70% methanol in PBSTx) prepared in *Step 7*. At each methanol concentration, incubate the glass vial on ice for 5 min.

To exchange solutions in the glass vial, carefully pour out the old solution and pipet in the new solution. The sample-attached filters will usually stick to the side of the glass vial at high methanol concentrations. At low methanol concentrations, use the cap of the glass vial to prevent pouring out the filter into waste.

10. After the last incubation in 70% methanol, transfer all filters into the pre-cooled 100% methanol in the other glass vial (see *Step 7*), and incubate the sample at –20°C for 10 min.

At this point, the dehydrated samples can be safely stored at –20°C for at least 3 months. For storage, clearly annotate the sample information and date on the screw cap using an alcohol-resistant marker pen.

11. Use a clean pair of forceps to take out desired samples on filters from the storage vial at -20°C , and transfer into a glass vial filled with 70% methanol in PBSTx. Rehydrate these samples using 4 mL of decreasing methanol concentration series (70%, 50% and 30% methanol in PBSTx) prepared in *Step 7*. At each methanol concentration, incubate the glass vial on ice for 5 min.

Hereafter the protocol assumes staining 2 different RNAs with 4 wild type E13 organs each. Therefore, take out 1 filter with 8 organs from the storage bottle, and keep the rest in methanol at -20°C for other experiments. The 8 organs will be detached from the filter and split into 2 groups (see *Step 16*).

12. After rehydration, rinse samples in the glass vial with 4 mL of PBSTx for 10 min at RT with gentle rocking on a laboratory rocker.
13. Further permeabilize samples in the glass vial using 4 mL of PBS-SDS for 20 min at RT with gentle rocking on a laboratory rocker.

Although methanol treatment may be sufficient for permeabilization, treatment with a strong detergent such as SDS can presumably destabilize interactions between RNAs and their bound proteins to make RNAs more accessible.

14. During SDS permeabilization, take out a clean 35 mm dish and a clean 24-well plate. In the 35 mm dish, fill with 3 mL of PBSTx and transfer 2 pre-cleaned sample baskets into this dish. Pipet 1 mL of Wash Solution into 2 new wells in the 24-well plate, and mark the sample information on the plate lid.

The choice of 24-well plate is because the diameter of its well is only slightly larger than the sample basket used here. You can adjust the plate type to match the size of your sample baskets.

15. After permeabilization, use forceps to transfer the filter with samples from PBS-SDS in the glass vial into PBSTx in the 35 mm dish. Under a dissection microscope, detach the 8 embryonic organs one by one from the filter using two pairs of forceps. For detaching, use one pair to hold the filter in position, and the other pair to gently insert between the organ and the filter.
16. After all samples have been detached from the filter, transfer 4 organs into each of the two sample baskets using a pair of bent-tip forceps. The bent-tip forceps can be self-made from worn dissection forceps that are no longer suitable for dissection. One tip should be bent inward towards the other tip, while the other tip should be bent upward perpendicularly to the plane of the two legs (Figure 2B). When transferring the sample, hold the bent-tip forceps underneath to scoop it up to avoid piercing it.

The horizontally bent tip touching the other leg forms a triangular frame that is smaller than the sample, whereas the vertically bent tip helps to hold the sample from the side. This bent-tip configuration thus makes it much easier to transfer samples than using regular forceps.

- 17.** After all samples are transferred into the sample baskets, transfer the baskets into the 24-well plate containing Wash Solution. Incubate for 10 min at RT with gentle rocking.

Note: The samples may float to the surface of the solution after the baskets are transferred into the 24-well plate. Gently tap the plate on a bench surface, or use a pair of blunt-ended forceps to gently push the samples into the solution. The samples should stay at the bottom of the basket once they are immersed in the solution.

- 18.** During the Wash Solution incubation, dilute 1 μL of smFISH probes (25 μM total) in 500 μL of Hybridization Solution to obtain a 50 nM working solution of smFISH probes for each of the two samples. Vortex the tube to mix, and then centrifuge the tube at 13,000 $\times$ rpm for 1 min to clear out bubbles.

A total final concentration of 50 nM smFISH probes ensures that each probe is within the optimal 1–2 nM, since each probe set contains 30–50 probes. This concentration works for RNAs expressed at a wide range of levels, and generally does not need to be varied.

Note: The Hybridization Solution is relatively sticky because it contains 10% Dextran Sulfate. Therefore, pipet slowly to ensure that an accurate volume is transferred.

- 19.** Pipet the probe solution into new wells next to the wells containing the sample baskets in the same 24-well plate, and then transfer the sample baskets into these wells. Wrap the plate with aluminum foil to protect samples from light. Incubate the foil-wrapped plate at RT for 15 min with gentle rocking to allow the sticky solution to equilibrate into the samples.

Note: It is important to protect samples from light for all subsequent incubations. Take care to minimize light exposure during buffer exchange and mounting.

- 20.** After this initial equilibration, use a pair of blunt-ended forceps to gently push the samples into the solution. Incubate the foil-wrapped plate in a humidified 37°C incubator without CO₂ for overnight (12–16 hours) to allow the smFISH probes to hybridize with their targets.

Note: Wipe the forceps with 70% ethanol between sample wells to avoid contamination.

Note: If the samples float up shortly after being pushed into the solution, leave the plate at 37°C for another 15 min, and push them into the solution again until they remain in the solution.

- 21.** (Day 2) The next morning, prepare 8 mL of Wash Solution according to the recipe listed under *Reagents and Solutions* enough for 4 washes for each sample. Thaw an aliquot of 50 $\mu\text{g/mL}$ DAPI stock solution for nuclear staining.

- 22.** Wash the samples 4 times with Wash Solution, each time for at least 30 min at 37°C. For the second wash, stain the nuclei with 0.5 µg/mL DAPI in Wash Solution for 2 hours at 37°C.

For washes, take out the foil-wrapped 24-well plate from the incubator, and pipet 1 mL of Wash Solution into 8 new wells next to the 2 sample wells. In each of the second set of wash wells, pipet in 10 µL of the 50 µg/mL DAPI stock solution for a final of 0.5 µg/mL DAPI. Transfer the sample baskets sequentially into the 4 sets of wash wells for washes and DAPI staining.

If you have enough wells in the 24-well plate, it is most convenient to line up all wash wells for the same sample in one row or one column. Otherwise, it is fine to exchange buffers by aspirating or pipetting from outside the sample baskets in the well. Be sure to use RNase-free pipet tips.

The samples tend to float up in the Wash Solution. When they do, gently tap the plate on the bench top, or use forceps to push the samples into the solution.

If nuclear staining by DAPI is sufficient for highlighting tissue architecture, there will be no need to do co-immunostaining. In that case, the samples can be rinsed in 2× SSC and mounted for imaging as described in Step 32 after the last wash.

- 23.** (Optional Co-immunostaining) During the last wash, prepare 1 mL of blocking solution by mixing 50 µL of donkey serum with 950 µL of 2× SSC (5% donkey serum in 2× SSC). Pipet 500 µL of the blocking solution into 2 new wells in the same 24-well plate, and transfer the sample baskets into the blocking solution. Block for 1 hour at RT with gentle rocking.

Blocking with serum from the same species as the secondary antibody greatly improves the signal-to-noise ratio during indirect immunostaining, but it does risk introducing RNases that may degrade RNAs in the sample. It is thus important to quality-control your reagents by comparing smFISH samples with or without the co-immunostaining. In my experience, 1 hour blocking with 5% of normal donkey serum (Jackson ImmunoResearch Laboratories) does not seem to affect the smFISH signal.

If you are staining highly expressed protein markers, it is possible to perform direct immunostaining without serum blocking, using fluorescently labeled primary antibodies. Alternatively, you may refer to Alternate Protocol 1 for a one-step co-immunostaining protocol using pre-mixed unlabeled primary antibodies and fluorescently labeled Fab fragments of secondary antibodies without serum blocking.

- 24.** Wash the samples twice with 1 mL of 2× SSC for 10 min at RT with gentle rocking.
- 25.** During the last wash, make 1 mL of primary antibody solution containing 1 µg/mL of rat anti-E-cadherin (Thermo Fisher, 13–1900) and 2 µg/mL of goat anti-collagen IV (MilliporeSigma, AB769) in 2× SSC.

- 26.** Incubate each sample in 500 μ L of primary antibody solution in the humidified 37°C incubator without CO₂ for overnight (16–20 hours).
- For whole-mount embryonic organs, overnight incubation at 37°C is required for the antibodies to fully penetrate into the center of epithelial tissue. Less incubation time is possible for staining protein markers in the surrounding mesenchyme.
- The sodium citrate in the 2 $\times$ SSC buffer has certain antibiotic effects and thus helps prevent bacterial growth. Consider adding 0.1% sodium azide if bacterial growth is observed.
- 27.** (Day 3) The next morning, wash the samples 4 times with 1 mL of 2 $\times$ SSC. For each wash, incubate at RT for 15 min with gentle rocking.
- 28.** During the last wash, make 1 mL of secondary antibody solution containing 2.5 μ g/mL of AF647 conjugated donkey anti-rat and 2.5 μ g/mL of Cy2 conjugated donkey anti-goat antibodies in 2 $\times$ SSC.
- 29.** Incubate each sample in 500 μ L of secondary antibody solution in the humidified 37°C incubator without CO₂ for overnight (16–20 hours).
- 30.** (Day 4) The next morning, wash the samples 4 times with 1 mL of 2 $\times$ SSC. For each wash, incubate at RT for 15 min with gentle rocking.
- 31.** During the washes, prepare the following to mount samples for microscopy imaging.
- 1.** Bring a dropper bottle of ProLong Diamond Antifade Mountant to room temperature, and place it upside down on a tube rack to help the sticky mountant reach the opening.
 - 2.** Pipet 3 mL of 2 $\times$ SSC into a clean 35 mm dish.
 - 3.** Cut the imaging spacers into small pieces (–8 pieces from each of the imaging spacer from Grace Bio-labs, Item # 654004). The imaging spacers are double-sided tape with a paper protector on one side and a plastic protector on the other side. Remove the paper protector to expose one sticky side, and then attach two imaging spacer pieces on a 75 $\times$ 25 mm glass slide, so that they occupy two corners across a diagonal line of a 22 $\times$ 22 mm area (see Figure 2C for a schematic). Peel off the plastic protector of the imaging spacer pieces to expose the other sticky side for the coverslip. Prepare one slide per sample group, and mark the sample information and date on the frosted annotation area of the slide.
- This geometry of imaging spacers is important for curing mounting media such as the ProLong Diamond Antifade Mountant used here. It provides enough support to avoid crushing of the samples by the coverslip, and at the same time allows the mounting media to make contact with air for successful curing.

Alternatively, the sample can be mounted in the enclosed space of a circular imaging spacer when using non-curing mounting media such as Fluoro-Gel (Electron Microscopy Sciences, Catalog Number 17985–31). However, samples mounted in Fluoro-Gel should be imaged as soon as possible after mounting (ideally within 1–2 days). Store Fluoro-Gel mounted samples at 4°C, and avoid freezing.

32. After the washes, follow instructions below to mount the samples one group at a time.

- 1.** Under a dissection microscope, transfer one sample basket from the 24-well plate to the 35 mm dish containing 3 mL of 2× SSC, and tilt the basket sideways to release all samples into the solution. Occasionally some samples may stick to the side of the basket, and you will need to use forceps to detach them. Remove the empty basket from the dish.

The sample baskets can be reused after being cleaned as described in step 3. Discard the sample basket when its bottom mesh is damaged or detached.

- 2.** From the dropper bottle, squeeze out a generous drop of ProLong Diamond Antifade Mountant inside the imaging spacer supported area on the glass slide. The exact amount does not matter, but should be enough to cover the whole coverslip area (–50 µL). Try to avoid bubbles.

If bubbles do appear, use a regular 20 µL pipet to suck them up. Add more mounting media if you removed too much when sucking up bubbles. Sometimes an unusually large amount of bubbles come out from the dropper bottle, and that is often a sign that the bottle is nearly empty.

- 3.** Use the bent-tip forceps to transfer samples of the same group one-by-one into the mounting media. When transferring, hold the sample from below using the bent-tip forceps (“scooping”) to avoid damaging the sample.

- 4.** After all samples in the same group are transferred into the mounting media, use the bent-tip forceps to push them down to touch the slide, and separate them using the forceps to avoid touching another sample.

- 5.** Place a 22 × 22 mm #1.5 thickness coverslip on top. Use forceps to hold one side of the coverslip. First, lay one side of the coverslip on the sticky surface of one piece of the imaging spacer, and then gently lower the coverslip down until the held side touches the other piece of the imaging spacer. Use the handle of forceps to press on and brush above the imaging spacers to firmly attach the coverslip.

When using ProLong Diamond Antifade or other mounts for oil objective microscopy, the #1.5 thickness of the coverslip does not

matter. However, when using Fluoro-Gel or equivalent mounting for using a water immersion objective, the thickness of the coverslip should match what the objective is corrected for (most likely for #1.5 coverslips).

6. Place the slide in a cardboard folder (or any dark container) to protect from light.
33. After all samples are mounted, leave the cardboard folder containing all slides at RT for at least 24 hours to allow the mounting media to cure. After curing, the slides can be stored at -20°C for at least several months. Refer to *Basic Protocol 2* for instructions of using fluorescence confocal microscopy to image the samples.

ALTERNATE PROTOCOL 1 (optional)

One-step Immunostaining by Pre-mixing Unlabeled Primary Antibodies with Monovalent Fab Fragment Secondary Antibodies

Introductory paragraph—The purpose of this protocol is to provide a simplified immunostaining strategy that does not require serum blocking, and requires less time. As mentioned in *Step 23* of *Basic Protocol 1*, blocking with serum from matching species of the secondary antibody can greatly improve the signal-to-noise ratio of typical indirect immunostaining, but it risks introducing RNases that may degrade RNAs in the sample. To skip serum blocking, you could use fluorescently labeled primary antibodies for direct immunostaining. Alternatively, you could pre-mix unlabeled primary antibodies with monovalent Fab fragment secondary antibodies. The key point here is to use a monovalent Fab fragment form of the secondary antibody (not F(ab')_2), so that pre-mixing primary and secondary antibodies does not generate aggregates. It is worth noting that both strategies will often result in dimmer fluorescent signals than canonical 2-step indirect immunostaining, so they are more useful for protein markers present at high concentrations. For demonstration of the protocol, I use E-cadherin staining as an example.

Materials

Reagents and Solutions

Rat Anti-E-cadherin Antibody (Thermo Fisher, 13–1900)

AF647 Fab Fragment Goat Anti-Rat IgG (Jackson ImmunoResearch, 112-607-008)

Ultrapure Water (e.g., Quality Biological, 351-029-131)

50% Glycerol in $1\times$ PBS (see recipe)

$2\times$ SSC (see recipe)

Sample Material

Mouse embryonic organs (E13) that have been processed through *Steps 1–22* of *Basic Protocol 1*.

Other Materials and Equipment:

Nuclease-free 1.6 mL Polypropylene Tubes (e.g., VWR, 4445.S.X)

50 mL Conical Tubes (Any Kind)

Parafilm

Laboratory Rotator

Humidified 37°C Incubator

Centrifuge for 50 mL Tubes

Aluminum Foil

Protocol steps—Step annotations

1. Reconstitute the lyophilized powders of antibodies, since both the rat anti-E-cadherin antibody and the AF647 Fab fragment goat anti-rat IgG come as lyophilized powder in glass vials.
 1. For the anti-E-cadherin antibody, add 200 μ L of 50% glycerol in 1 $\times$ PBS into the glass vial containing 100 μ g antibody to obtain a 0.5 mg/mL stock solution (note that lyophilized powder contains salt and carrier BSA protein). Put the screw cap back on the vial, seal with parafilm, and rotate on an end-over-end laboratory rotator for 5–10 min at 30–60 rpm to fully dissolve the powder.
 2. For the AF647 Fab fragment goat anti-rat IgG, add 1000 μ L of 50% glycerol in 1 $\times$ PBS into the glass vial containing 500 μ g antibody to obtain a 0.5 mg/mL stock solution. To fully dissolve the powder, put the rubber stopper back on the vial, seal with parafilm, and rotate on an end-over-end laboratory rotator for 5–10 min at 30–60 rpm.
 3. Centrifuge the glass vials to bring all solution to the bottom for transfer. To centrifuge the glass vials, first place some paper towel in a 50 mL tube as a cushion, then insert the glass vial with the cap upward, and finally put in more paper towel to hold the glass vial in position. Balance the two tubes by putting in more or taking out some paper towel material, and centrifuge at 1,000 $\times$ g for 1 min.
 4. Transfer the antibody solution into a clean 1.6 mL tube, mark the information (identity, concentration and date) on the tube, and store at -20°C . Note that the stock solution with 50% glycerol in 1 $\times$ PBS will not freeze at -30°C .
2. After the final wash with Wash Solution (Step 22 of Basic Protocol 1), rinse the samples once with 2 $\times$ SSC for -30 min at RT with gentle rocking on a laboratory rocker.
3. During the 2 $\times$ SSC wash, mix 4 μ L of the reconstituted 0.5 mg/mL rat anti-E-cadherin antibody plus 4 μ L of the reconstituted 0.5 mg/mL AF647 Fab fragment

goat anti-rat IgG into 1 mL of 2× SSC in a 1.6 mL tube. Wrap the tube with aluminum foil, and rotate for 30 min at 30–60 rpm to allow binding of the primary antibodies to the labeled Fab fragment secondary antibodies.

Because intact IgG has a molecular weight of ~150 kDa, while the Fab fragment is ~50 kDa, using equal mass concentrations of the two yields a molar ratio of 1:3 for primary antibody IgG to the labeled fragment. Thus, each IgG-Fab complex will have on average 3 fluorescently labeled Fab fragments, resulting in a higher fluorescent labeling ratio than most direct labeling of primary antibodies.

4. Remove the 2× SSC from the sample wells, and add 500 µL of pre-mixed antibodies. Incubate the sample plate for overnight at 37°C.
5. The next morning, wash and mount the samples as described in *Steps 30–33* of *Basic Protocol 1*.

SUPPORT PROTOCOL 1 (optional)

Enzymatic Labeling and Purification of smFISH Probes

Introductory paragraph—Although it is convenient to order ready-to-use smFISH probes from LGC Biosearch Technologies, it can be costly. Recently, a low-cost enzymatic labeling method has been reported (Gaspar et al., 2017) that uses terminal transferase to catalyze the controlled addition of only one fluorescently labeled ddNTP (dideoxynucleotide triphosphate) to the 3'-end of unlabeled oligo probes. I have adopted this method, and have demonstrated that custom-labeled probes using this method perform equally well as commercial probes (Figure 3). The protocol below describes the steps to label and purify smFISH probes from unlabeled DNA oligos.

Materials

Reagents and Solutions:

Unlabeled smFISH Probes (See *Step 1*)
Amino-11-ddUTP (1 µmol scale; Lumiprobe, 15040)
100 mM NaHCO₃, pH 8.30 (see recipe)
Ultrapure Water (e.g., Quality Biological, 351-029-131)
Atto-655 NHS Ester (1 mg scale; Sigma-Aldrich, 76245-1MG-F)
DMSO (e.g., Sigma-Aldrich, D2650)
1 M Tris Buffer, pH 7.40 (e.g., K D Medical, RGF-3340)
0.5 mM TMR Conjugated ddCTP (Jena Biosciences, NU-850-TAM)
Terminal Transferase Kit (New England Biolabs, M0315L)
3 M Sodium Acetate, pH 5.20 (e.g., Quality Biological, 351-035-721)
Ethanol

75% Ethanol

5 mg/mL Linear Acrylamide (e.g., Thermo Fisher, AM9520)

Equipment

8-Channel or 12-Channel Multichannel Pipette

Pipette Tips Compatible with the Multichannel Pipette

Disposable Reagent Reservoir for Multichannel Pipette (e.g., Thermo Fisher, 8096–11)

Laboratory Rotator

Humidified 37°C Incubator (without CO₂)

Vortex Mixer

Refrigerated Centrifuge

Nanodrop Spectrometer (or a regular spectrometer)

Protocol steps—Step annotations

1. Design smFISH probes as described in *Step 1 of Basic Protocol 1*. Order the designed probes as individual DNA oligos from your preferred oligo supplier, such as Integrated DNA Technologies (IDT).

It is preferred to order oligos to be delivered at normalized 100 μ M concentrations in a 96-well or 384-well plate format to accelerate oligo mixing using multichannel pipettes. In addition, the plate layout should be designed to cluster oligos of the same probe set in rows or columns to make the best use of your multichannel pipette.

For demonstration of this protocol, an 8-channel multichannel pipette was used for two smFISH probe sets, each having 48 probes. Thus, each probe set was placed in 6 columns of a 96-well plate such that probe set 1 occupies A1–H1, A2–H2 ... A6–H6, whereas probe set 2 occupies A7–H7, A8–H8 ... A12–H12.
2. Once the unlabeled probes arrive from the supplier as 100 μ M solutions in a 96-well plate, use the multichannel pipette to transfer 20 μ L each of the 48 probes in the same probe set into a disposable reagent reservoir. Mix well in the reservoir, and transfer the mixed probe solution into a clean 1.5 mL tube. Keep the mixed probe oligos (100 μ M total) frozen at –20°C for long-term storage.
3. Prepare Atto-655 conjugated ddUTP.
 1. To the amber glass vial of Atto-655 NHS ester (1 mg), add 28.3 μ L of DMSO to obtain a 40 mM final concentration of Atto-655 NHS ester solution.
 2. To the tube of amino-11-ddUTP (1 μ mol), add 100 μ L of 100 mM NaHCO₃ (pH 8.30) to obtain a 10 mM final concentration of amino-11-ddUTP.

Note: proper pH of the buffer is critical for the NHS ester and amine reactions.

3. Add 56.50 μL of the 10 mM amino-11-ddUTP into the amber glass vial containing 28.25 μL of 40 mM Atto-655 NHS ester solution. Wrap the lid with parafilm, and rotate on an end-over-end laboratory rotator for 2–3 hours at RT.
4. Add 28.25 μL of 1 M Tris buffer (pH 7.40) to quench the excess NHS ester. Rotate end-over-end on a laboratory rotator for an additional 30 min at RT.
5. The final Atto-655 conjugated ddUTP concentration is 5 mM. Pipet into 10 μL aliquots, and store frozen in dark at -20°C .

4. In a nuclease-free 1.5 mL tube, set up tailing reactions using TMR-conjugated ddCTP by mixing (in order) 24 μL of ultrapure water, 10 μL of 100 μM mixed probe oligos (*Step 2*), 4 μL of 0.5 mM TMR-conjugated ddCTP (2 $\times$ molar excess), 5 μL of the 10 $\times$ Reaction Buffer, 5 μL of the 2.5 mM CoCl_2 , and 2 μL of the 20 U/ μL terminal transferase. Note that the last three components are all from the NEB Terminal Transferase kit.

If using Atto-655 conjugated ddUTP for probe labeling, use 27 μL of ultrapure water and 1 μL of the Atto-655 conjugated ddUTP (5 $\times$ molar excess).

5. Incubate the reaction at 37°C overnight.

If using TMR-conjugated ddCTP for labeling, 1.5 hours is sufficient, but overnight is also fine. For Atto-655 conjugated ddUTP, I have only tried overnight reactions.

6. (Day 2) The next morning, chill 50 mL of 100% ethanol and 50 mL of 75% ethanol at -20°C .

7. Take out the tailing reactions from 37°C . To each tube, add 130 μL of ultrapure water, 20 μL of 3 M sodium acetate (pH 5.20), and 1 μL of 5 mg/mL linear acrylamide. Mix well by vortexing, then add 800 μL of pre-cooled ethanol and mix again. Incubate the mix on ice for 30 min to a few hours to precipitate DNA oligos.

Note: Linear acrylamide serves as a carrier for oligo precipitation. It is OK to leave out, but the recovery rate will be slightly lower.

8. Centrifuge at 16,000 $\times$ g at 4°C for 20 min to pellet the oligos.

At this step, a colored pellet (red for TMR labeling; cyan for Atto-655 labeling) should be visible at the bottom of the tube.

9. Decant the supernatant and wash the pellet by adding 1 mL of cold 75% ethanol and vortex to resuspend the pellet. Centrifuge pellet again at 16,000 $\times$ g at 4°C for 20 min.

10. Decant the supernatant, and centrifuge the tube briefly at 16,000× g for 1 min (temperature does not matter) to centrifuge the residual liquid to the bottom of the tube. Use a 100 µL pipette to carefully remove the residual liquid without disturbing the colored pellet. Air dry the pellet by leaving the tube open on bench in a clean area for 10–15 min.
11. Resuspend the pellet using 50 µL ultrapure water to obtain purified labeled probes at a concentration of about 20 µM.
12. Pipet out 1 µL of the labeled probe solution to measure the concentration and degree of labeling (DOL) using a nanodrop spectrometer (or any appropriate spectrometer you have). Record the absorbance values at the peak absorption wavelength of the DNA oligo (260 nm; denoted A_{260}) and at the maximum absorption wavelength for the fluorescent dye (553 nm for TMR, 663 nm for Atto-655; denoted $A_{\text{dye_max}}$).
13. Calculate the molar concentration of the labeled probe solution (c), the DOL and the recovery rate using the following equations:

$$c = (A_{260} - CF_{260} \times A_{\text{dye_max}}) / \epsilon_{\text{oligo}}$$

$$\text{DOL} = A_{\text{dye_max}} / \epsilon_{\text{dye}} / c$$

$$\text{recovery rate} = c \times 50 \mu\text{L} / n_{\text{start}}$$

1. A_{260} and $A_{\text{dye_max}}$ are the absorbance values measured in *Step 12*.
2. CF_{260} is the correction factor at 260 nm of the fluorescent dye: TMR, $CF_{260} = 0.32$; Atto-655, $CF_{260} = 0.24$. This is because the incorporated fluorescent dye will increase the absorbance at 260 nm to some extent.
3. ϵ_{oligo} is the average molar extinction coefficient of the probe oligos. For 21 nucleotide long oligos (20 plus incorporated ddU or ddC), it is usually around $2.0 \times 10^5 \text{ Lmol}^{-1}\text{cm}^{-1}$, which is sufficiently precise for the concentration calculation. Alternatively, the Thermo Fisher multiple primer analyzer can be used to calculate the average ϵ_{oligo} : <https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html>. Then add 9000 to account for the incorporated ddU or ddC.
4. ϵ_{dye} is the molar extinction coefficient of the used fluorescent dye: TAMRA, $\epsilon_{\text{dye}} = 7.8 \times 10^4 \text{ Lmol}^{-1}\text{cm}^{-1}$; Atto-655, $\epsilon_{\text{dye}} = 1.25 \times 10^5 \text{ Lmol}^{-1}\text{cm}^{-1}$.

5. The DOL should be close to 1.0. Because a terminator nucleotide ddUTP or ddCTP is used, the theoretical upper limit of DOL is also 1.0.

For Atto-655-ddUTP labeling, I routinely obtain a DOL of 0.9–1.0. For TMR-ddCTP labeling, I routinely observe an inflated 1.2–1.4 DOL that probably reflects the altered property of DNA conjugated fluorescent dye, or the co-precipitation of un-incorporated TMR-ddCTP in the purification process. This does not affect the performance of the probes.

6. n_{start} is the amount of starting oligos used for labeling, here I used 10 μL of 100 μM concentration, so $n_{\text{start}} = 10 \mu\text{L} \times 100 \mu\text{M} = 1 \text{ nmol}$. In this case, if the measured concentration of the probe solution is 17 μM , the recovery rate will be: $17 \mu\text{M} \times 50 \mu\text{L} / 1 \text{ nmol} = 85\%$.

14. Pipet the purified, fluorescently labeled smFISH probe solutions into 10 μL aliquots, and store frozen at -20°C .

BASIC PROTOCOL 2 (optional)

Fluorescence Confocal Microscopy Imaging of smFISH Samples

This protocol describes the steps taken to image whole-mount mouse embryonic organ samples stained by smFISH using fluorescence confocal microscopy. Although confocal microscopy was previously thought to be unsuitable for smFISH imaging (Ji and van Oudenaarden, 2012), it is required in this procedure to reject out-of-focus light for imaging through thick tissues. I have successfully used laser scanning confocal fluorescent microscopy for imaging to 40–60 μm depths in various samples including mouse embryonic salivary glands, lungs and kidneys (Figure 1B).

It is important to realize that the smFISH signal is much harder to detect than regular immunostaining signals due to the intrinsically low signal from each transcript (30–50 fluorophores are expected) and its sub- or near-diffraction limit size. To ensure robust detection of smFISH spots, a high numerical aperture (NA) objective should be used to ensure sufficient optical resolution. In addition, the pixel size should be set to be smaller than half of the optical resolution to ensure sufficient image sampling (look into the concept of Nyquist sampling; e.g., the Wikipedia pages of Nyquist rate and Nyquist–Shannon sampling theorem) – this requirement is often not necessary when imaging immunostaining samples.

Materials

ProLong Diamond Antifade Mounted Slides from *Basic Protocol 1*

For demonstration here, E13 salivary glands stained with DAPI, Cy2-labeled secondary antibodies for anti-collagen IV (basement membrane marker), TMR labeled Cdh1 smFISH probe (Cdh1 is the gene encoding E-cadherin, an epithelial junction marker), and AF647-labeled secondary antibodies for anti-E-cadherin, were used.

Laser Scanning Confocal Fluorescent Microscope System Equipped with 4 Laser Lines (405 nm, 488 nm, 561 nm, and 633 or 640 nm)

I have successfully used both a Nikon A1R confocal laser microscope system (e.g., images in Figures 1B, 3B) and a Zeiss LSM 880 with Airyscan system for smFISH sample imaging (e.g., images in Figures 1C–D). The following step by step instructions are for the Nikon A1R system that is controlled by the NIS-elements AR software. Any equivalent laser scanning confocal microscope system should work.

Protocol steps—Step annotations

1. Bring the slide for imaging from -20°C to RT.
2. To locate samples under the microscope, use a marker pen to draw a small circle around each mounted organ on the back side of the slide (the coverslip side is the front side).

Note that the drawn circle will not interfere with fluorescent microscopy imaging, because both the excitation and emission light goes through the objective, which is on the coverslip side.

3. Follow the standard operation procedure of your microscope instruction to turn on the laser scanning confocal fluorescent microscope system.
4. Switch to a high NA oil objective suitable for fluorescent imaging (e.g., the Nikon Plan Apo 60 $\times$ oil objective, NA 1.4). Carefully clean the objective using lens paper.
5. Add immersion oil, and load the sample slide into a slide holder on the microscope stage.

If using an inverted microscope, lower the objective first using the focus knob to prevent the slide from initially directly contacting it. Second, add a generous drop of immersion oil to the center of the objective. Third, lock the sample slide into the slide holder with the coverslip facing down. Finally, carefully raise the objective until the immersion oil touches the coverslip.

It is very important to avoid introducing bubbles when adding immersion oil and adjusting the objective to touch the coverslip. If you notice bubbles, remove the slide, wipe off the immersion oil using lens paper, and try it again.

6. Locate your samples for imaging.
 1. Redirect light to the eyepiece. In the NIS-elements AR software, click the “Eye Port” button in the “A1plus Compact GUI” panel. Use the white light instead of epifluorescence for locating your sample to minimize photo bleaching.
 2. Roughly center the sample by moving around the slide with the stage controller. Use the drawn circle on the back of the slide for reference.
 3. While looking through the eyepiece, adjust the focus knob to focus on the sample. Slowly move around the slide to center at the positions you

want to image in the view field. Record the stage positions in the microscope control software (e.g., NIS-elements AR).

Most modern microscope systems allow users to record multiple stage positions. If you plan to image multiple positions for several samples on the same slide, it is easier to locate and record the position of each sample when you have light through the eyepiece.

For each E13 salivary gland, I typically image 1–3 regions at each region including the periphery of epithelial buds, the inner epithelial buds, the mesenchyme, the secondary ducts and the primary duct. Different organs have different architectures, but it is usually a good practice to image at least 1 position per structurally distinct region.

7. Fine-tune stage positions using DAPI-stained nuclei and co-immunostained protein marker. This step is necessary because the stage positions determined by looking through eyepiece under white light are often not very precise. Here I use a combination of the DAPI staining (405 nm excitation) and E-cadherin immunostaining (640 nm excitation) as the structural reference to fine-tune stage positions.

1. Redirect light to the detectors. In the NIS-elements AR software, click the “Eye Port” button again will do this.
2. Set image acquisition parameters using the lowest practical levels of light intensity possible for adjusting stage positions to reduce photo bleaching.

Typically, I use 1% power for the 405 nm laser and 5% power for the 640 nm laser (100% = 15 mW for both). The PMT (photomultiplier tube) gain of both detectors are set to 120 with 0 offset. The pinhole size is set at 1.2 AU (Airy unit). The scanning frame size is set to 512×512 pixels. The bidirectional scanning mode of the galvano scanner is used to double the scanning speed. No line average is used. The two channels are simultaneously scanned, since the 405 nm and 640 nm channels are sufficiently far apart so that there is no crosstalk or bleed-through issues.

3. Activate the live scanning mode, and finely adjust the x, y and z at each recorded stage position to the intended region of interest using DAPI-stained nuclei and immunostained E-cadherin as tissue landmarks. Modify the recorded stage position after each adjustment.

8. Set image acquisition parameters to meet your research needs.

1. For most cases, I keep the pinhole size at 1.2 AU.

You can vary the pinhole size between 1.0 to 1.5 AU. Smaller pinhole size gives better resolution especially in the axial direction, but at the cost of reducing signal intensity. In my experience, 1.2 AU is usually a good balance.

2. Adjust the confocal zoom and scanning frame size to set the pixel size smaller than the half size of the lateral resolution of your microscope system.

For example, when using a 60× NA 1.4 objective, 1.2 AU pinhole size and 561 nm excitation wavelength, the lateral resolution is about 130 nm. In this case, I often use a combination of 2× confocal zoom with 2048 × 2048 frame size, or 4× confocal zoom with 1024 × 1024 frame size to make the pixel size about 50 nm.

3. Optimize image acquisition by adjusting the laser power, pixel dwell time, PMT gain and offset using the image histogram as a reference.

It is important for microscope users to become familiar with the image histogram, which most modern microscope systems provide as a reference for adjusting imaging parameters. A typical image histogram uses the pixel intensity values as its x axis, and the total number of pixels with the corresponding intensity value as its y axis. The y-axis sometimes uses a non-linear scale (e.g., log scale) to facilitate inspection of near-zero values.

When adjusting image acquisition parameters, make sure the image is not over-saturated or under-saturated. In the histogram, over-saturation and under-saturation display as a truncated distribution to the right or left, respectively. If the sample is sufficiently bright, it is ideal to make the range of histogram distribution span a large range of pixel intensity values (e.g., a few thousands when the image depth is 12-bit). While the DAPI staining and immunostaining channels are often bright enough for this adjustment, smFISH staining channels are often the dimmest. In practice, I often end up setting the pixel intensity range of TMR-labeled smFISH channel to be 200–300 gray scale values.

In the Nikon A1R system, I usually use the default smallest 1.1 μs pixel dwell time, and keep the PMT offset at 0 unless the image is under-saturated. A good starting point of the other two acquisition parameters for each channel is listed:

1. DAPI channel: 405 nm laser power 1%, PMT gain = 120
2. Cy2 immunostaining of collagen IV: 488 nm laser power 2%, PMT gain = 50
3. TMR smFISH: 561 nm laser power 10%, PMT gain = 55
4. AF647 immunostaining of E-cadherin: 640 nm laser power 5%, PMT gain = 120

Note that all 4 laser lines used here has 15 mW output power at the fiber end.

4. Set the number of z-slices and z-step size depending on your research needs.

It is important to consider whether you need a single z-slice image or multiple z-slice images. For example, if the purpose of your experiment is to get an overview of the target RNA expression pattern, a single z-slice image at multiple positions will be sufficient. If the expression pattern at different z-levels may differ, consider acquiring multiple z-slice images at relatively sparse z-intervals (2–5 μm) to cover a large volume. Lastly, if it is important for you to capture every single dot within a z-range, you will need to acquire multiple z-slice images at a very small z-interval that corresponds to the half size of the axial resolution of your imaging system (0.2–0.5 μm). In the last scenario, however, it is important to realize that photo bleaching will make it only practical to sample a z-range of 10–30 μm (e.g., image in Figure 1D covers a $\sim 8 \mu\text{m}$ z range).

5. Set the scanning line average to be 2 $\times$, 4 $\times$, 8 $\times$ or 16 $\times$, depending on the signal-to-noise ratio of your sample, and how many z-slices need to be acquired.

Use of line average helps smoothen the image background at the cost of increased imaging time and photo bleaching. If you are acquiring only one z-slice image at each position, you can maximally increase the number of line averages, since photo bleaching is not as much of a concern. However, when acquiring multiple z-slices especially at small z-step size, photo bleaching can severely affect your image quality, and you may want to minimize the number of line averages. In practice, I often use 8 $\times$ or 16 $\times$ line average when acquiring single-z images, but only 2 $\times$ or 4 $\times$ when acquiring multiple z-slices.

To explain the principle, let's assume the background pixel intensity has an intrinsic variation of v , when the sample is scanned for one time. When $n\times$ line average is used, the sample is scanned n times, and the average of these scans is taken as the intensity at each pixel. In this case, the variation of the background pixel intensity is v divided by the square root of n . You can look into the relationship of SEM (standard error of the mean) and SD (standard deviation) in statistics for further understanding.

6. Set up the channel series of scanning to avoid fluorescence signal bleed-through.

The excitation and emission spectra of the fluorescent dyes used, the emission filters used in the microscope system, and the relative brightness of each channel collectively determine whether simultaneous acquisition of different channels is possible without causing bleed-through issues. To avoid fluorescence signal bleed-through in our

system, I use 3 passes of scanning for 4-channel acquisition, where only the 405 nm and 640 nm channels are scanned simultaneously.

9. Start imaging acquisition of all recorded stage positions. For image analysis, refer to *Basic Protocol 3*.

BASIC PROTOCOL 3

Semi-Automated smFISH Image Analysis Using ImageJ Macros

The microscopy images from a successful smFISH experiment should show a certain number of clearly visualized fluorescent dots above the background. Each dot presumably represents one molecule of the target RNA. The basis of smFISH image analysis is to count the number of dots within a region of interest (ROI). There are usually two steps to implement an automated dot counting strategy. In the first step, the images are subjected to a series of image filters for reducing potentially non-uniform background while enhancing the contrast of dots at a chosen size. In the second step, the image is binarized, the dots are segmented and counted. Raj et al. have implemented semi-automated smFISH image analysis in MATLAB (Raj et al., 2008) by a Laplacian of Gaussian filter (i.e., LoG filter) followed by thresholding the filtered image using a custom-chosen threshold level. Here, I implement a similar strategy using the open-source ImageJ distribution termed Fiji (Schindelin et al., 2012) using a morphological top-hat filter (Legland et al., 2016) followed by a combination of local maxima finding and thresholding. This protocol describes detailed instructions for using the provided ImageJ macros to perform semi-automated smFISH image analysis.

Materials

Microscopy Images of smFISH (test images provided in Supplemental Material)

Fiji Software Installed on a Mac or Windows Computer (see *Step 1 and 2* for installation and configuration)

Custom-written ImageJ Macros (Supplemental Material)

Protocol steps—Step annotations

1. Download and install the Fiji software from <https://fiji.sc> for your computer system.

Fiji is a distribution of ImageJ that comes with many useful plugins for biological image analysis. Importantly, the core “Bio-Formats” plugin enables Fiji to open most commonly-used microscopy image formats, including various “tiff” formats, the MetaMorph “stk” format, the Nikon “nd2” format, the Zeiss “czi,” lsm,” and “zvi” formats, and many more.

2. Configuration of essential plugins for this analysis:

1. Configure the Bio-Formats plugin for your image format.

For image formats with meta-data, “Bio-Formats” often displays a dialog window to allow the users to customize the information to show when opening the image. While this dialog is very useful to extract extra meta data information or specify special display options, it unnecessarily disrupts the automated workflow by forcing users to at least click once when opening each image. The configuration here is to suppress the display of this dialog.

Click through the menu: Plugins -> Bio-Formats -> Bio-Formats Plugins Configuration. A dialogue window with the title “Bio-Formats Plugins Configuration” will show up. This dialogue window has 4 tabs: “General,” “Formats,” “Libraries,” and “Log.” Activate the “Formats” tab. To the left of the panel there is a long list of supported formats. Go through to find the format of your images. For example, the supplied 2D test images use the Nikon ND2 format and have “.nd2” extensions. Select “Nikon ND2” from the list on the left. On the right, a few checkboxes will appear. Make sure to check “Enabled” and “Windowless” (Figure 4A).

2. Install the MorphoLibJ plugin by enabling the “IJPB-plugins” update site.

Click through the menu: Help -> Update... to check and download Fiji updates. At the end of update checking and downloading, a dialog with the title “ImageJ Updater” will appear, where the downloaded updates are listed (could be empty; Figure 4B). Click on the button “Manage update sites” towards the lower left corner of the dialog window, which will bring up another dialog with the title “Manage update sites” (Figure 4B). Go through the listed sites, and check the “IJPB-plugins” update site to enable automatic downloading of the MorphoLibJ plugin (Figure 4B). Close the “Manage update sites” dialog, and the downloaded MorphoLibJ plugin should be listed in the “ImageJ Updater” list (Figure 4B). Click on “Apply changes” to install all downloaded updates. Restart Fiji after updating. If you have successfully installed the MorphoLibJ plugin, you should now have an additional item “MorphoLibJ” under the “Plugins” menu.

3. Estimate the smFISH dot size using “smFISH-estimateDotRadius.ijm.”

1. In Fiji, run the supplied macro “smFISH-estimateDotRadius.ijm.”

There is more than one way to run a macro file. First, you can click through the menu: Plugins -> Macros -> Run..., which will open a dialog asking you to navigate the folders to find the macro file. Second, you can drag the macro file onto the Fiji menu bar, which will open the macro script editor (see image in Figure 4C, Step 1). Towards the bottom left of the script editor, there is a “Run” button you can click to run the macro.

2. The macro will open a dialog window asking you to choose a file for this task. Navigate to any smFISH image file in your image folder to open it.

Once an image is chosen, the folder containing this image is considered as the input folder, and the macro will create an output folder inside the same parental folder as the input folder, with the input folder name plus an “-output” postfix. All output files and log files will be saved into the output folder.

3. The macro will then prompt the user to specify how many dots to go through for diameter calibration. The default is 5, but you can use more for calibration if you want to spend more time.

4. The macro will then open the image, and loop for 5 times (or however many times as the number of dots you specified) for you to draw a line segment across the diameter of 5 randomly chosen dots. Make sure the line segment is drawn from edge to edge within the dot. The average radius will be calculated and converted to pixel unit. Record this number (or numbers for 3D images) to use later in *Step 4-(3)*.

In practice, the dots are often oblong instead of perfectly circular due to the suboptimal configuration of the microscope system. When drawing line segments across these dots, avoid either the longest or shortest axis, but rather aim for a middle ground.

I obtained an average pixel radius of 4 when I randomly measured 5 dots in a test image. This value will be input as the radius of the morphological top-hat filter for image filtering (see next step).

For 3D images, the macro will measure the radius in both xy and z directions. For each dot, it will first go to the middle slice of the image, and then prompt the user to draw a line across any clear dot with the line extending beyond the dot. The macro create a so-called “re-slice” image, where each row of pixels corresponds to the pixels along the line on a z-slice. Second, the macro will prompt the user to draw a horizontal line (xy direction) across the dot to measure the diameter in xy direction. Third, it will prompt the user to draw a vertical line (z direction) across the dot to measure the diameter in z direction. A different dot should be measured at each prompt until the specified number of dots have been measured. In the end, the macro displays calculated dot radius in xy and z directions in pixel (or voxel) units.

4. Threshold parameter optimization using “smFISH-thresholdOptimization.ijm.”
 1. In Fiji, run the supplied macro “smFISH-thresholdOptimization.ijm” either through the menu or the Fiji script editor as described in *Step 3-(1)*.

As briefly mentioned in the introduction, the macro will loop through all possible threshold values of the filtered image, and prompt the user to determine the best threshold level. Because this is computationally expensive and time consuming, and because a similar threshold level is often chosen for images with similar signal-to-noise-ratio (e.g., images from the same set of experiments), a small number of images can be used to determine the optimal threshold level, which will then be used for the entire set of images.

On the other hand, if you don't have many images, you can use this macro to process all images (in place of Step 5).

2. The macro will first prompt the user to navigate to the input folder containing the images you intend to use for threshold parameter optimization (Figure 4C, step 1).

If you have run “smFISH-estimateDotRadius.ijm,” an output folder should have been created to save all intermediate and output files. If you skipped that one because you knew the dot radius, running this macro will create an output folder inside the same parental folder as the input folder, with the input folder name plus an “-output” postfix.

3. Next, the macro displays a dialog to ask for a few parameters for image filtering (Figure 4C, step 2). There are 3 mandatory parameters, 1 additional parameter for multi-channel images, 2 additional parameters for 3D images, and 2 optional checkbox options. They are quite self-explanatory on the user interface. Nonetheless, here I will go through them one by one:

1. “How many files do you want to use for threshold optimization?” Specify the number of images you want to go through for threshold optimization.
2. “Gaussian Blur Radius (xy) in pixels.” This parameter is used to smoothen the image. If your image is sufficiently sampled, or the pixel size is smaller than half of the optical resolution, you can leave this number as the default “1” pixel. Otherwise, if your image is not sufficiently sampled, reduce this number “0” to turn off smoothening.
3. “Morphological Top-hat Radius (xy) in pixels.” This parameter is used to enhance the contrast of objects with the specified radius. Therefore, use the average dot radius recorded from *Step 3-(4)*.
4. (For multi-channel images) “Which channel to process?” When you have multi-channel images, you need to specify which channel is the smFISH channel to process. In case you have multiplexed smFISH channels, you will need to process one channel at a time. After processing the first smFISH

channel, remember to change the output folder name so that the files will not be over-written.

5. (For 3D images) “Gaussian Blur Radius (z) in voxels.” This additional parameter is used to smoothen the image in z-direction. If your z-step is large compared to the dot radius in z-direction, use “0” here to filter each z-slice as a 2D image. Otherwise, use “1” or “2.”
6. (For 3D images) “Morphological Top-hat Radius (z) in voxels.” This additional parameter is used in conjunction with the “Morphological Top-hat Radius (xy) in pixels” to enhance the contrast of 3D ellipsoid objects. If your z-step is large compared to the dot radius in z-direction, use “0” here to filter each z-slice as a 2D image. Otherwise, use the calibrated dot radius in z-direction.
7. “Keep intermediate files” checkbox. When checked, all intermediate files are kept to greatly speed up repeated manual selection of threshold.

Tip: The “Keep intermediate files” checkbox can be repurposed for deleting intermediate files in the temporary folders. Just put “0” in the “How many files do you want to use for threshold optimization?” option, uncheck the “Keep intermediate files” checkbox, and click OK to run the macro – it will take only 1 second to remove all temporary folders in the output folder.
8. “Show results one-by-one after processing all” checkbox. When checked, three output images at the chosen threshold will be displayed after processing all files for threshold optimization (Figure 5B). Leave it unchecked unless you want to double-check your selection for each file.
4. After clicking “OK” on the parameter dialog, the macro will remain silent for a while depending on the size and number of files you placed in the folder (see *Time Considerations* for typical processing time). Behind the scene, the macro is looping through each file to filter them, threshold at all possible levels, and prepare intermediate image files for display to the user in the next step.
5. After pre-processing all image files from the input folder, the macro will present to the user sequentially a plot and a set of images to help the user select the optimal threshold level.

There are two steps of user interaction. In the first step, a plot is displayed. This plot has the normalized dot number in both linear and log scales as its y axis, and the corresponding threshold level as its x axis. The plot curve of a successful smFISH image with clear dots over

the background will always have a kink (inflection) point followed by a partial plateau (Figures 4C, step 3). This partial plateau marks the threshold region where the identified dot number is relatively insensitive to the threshold. When the partial plateau is obvious, the middle point of the partial plateau region typically corresponds to the optimal threshold level. Point your mouse cursor near the middle of the partial plateau on the curve, and click the left mouse button. The macro will record this selected position and use it as the initial point for visual inspection.

In the second step, the original image, the filtered image, and the “dots vs. threshold” image stack are displayed side-by-side to help the user determine the optimal threshold level while going through the dots-vs-threshold image stack. At this step, the Fiji tool “Synchronize Windows” is especially helpful. After clicking the “Synchronize All” button, all displayed windows will show a red cross-shaped cursor at matching image locations, which can be used to inspect the quality of segmented dots at the selected threshold (Figure 4C, step 4).

For 3D images, a similar interface is presented for threshold optimization, but the displayed images are considerably different. To facilitate quality assessment, I use a tile of 3 maximum intensity projection images in the 3 windows showing the original image, the filtered image and the dots-vs.-threshold image stack (Figure 5C). The top and middle tile are maximum intensity projection of a z-range equivalent to a dot diameter in z-direction centered at 1/3 and 2/3 of the entire z-range, whereas the bottom tile shows the maximum intensity projection of all z-slices.

When you are satisfied with the choice, click OK to record the selection and continue. The macro will then extract the segmented dots image at the selected threshold level from the intermediate results.

When the specified number of files are looped through, a summary of selected threshold levels will be displayed. This summary will serve as a reference for you to choose one threshold level that will be used to quantify all images from the same experiment (e.g., control and experimental groups). Typically, you may use the mean of manually selected threshold levels, but it is up to you to use a higher or lower threshold, depending on how conservative or aggressive you want to be in identifying individual dots. Make a note of this chosen threshold for use in the next step.

5. Run all images from the same experiment (e.g., stained with the same probe and imaged using identical acquisition parameters) at the selected threshold using “smFISH-processAll.ijm.”

The beginning parameter interface of this macro (Figure 5A) is very similar to that of “smFISH-thresholdOptimization.ijm” with 3 small differences.

First, the dialog does not ask for the number of files to process, since all files in the input folder will be processed.

Second, the dialog asks for one more parameter “Selected Threshold Level.” Input the chosen threshold level from Step 4-(5). This threshold level will be applied to all images from the input folder for dot identification.

Third, the “Keep intermediate files” checkbox is replaced by “Count dots inside and outside specified ROI?” When this checkbox is checked, the macro will count the number of dots inside and outside of the ROI when a matching ROI file is found in the “ROIs” folder inside the output folder. A matching ROI file needs to have the same file name with the input image file followed by “.roi” for 2D images and “-ROIset.zip” for 3D images.

The ROI files can be generated by manual drawing when dealing with a small number of 2D images. The provided “smFISH-drawROI-2D.ijm” macro can be used to reduce the work of manual drawing. However, manual drawing is often tedious or impractical for a large number of images, or 3D images with considerable number of z-slices. In those cases, co-immunostained protein markers can often be used to automatically generate meaningful ROIs, but the strategy needs to be developed on a case-by-case basis.

6. The output files of the “smFISH-thresholdOptimization.ijm” and “smFISH-processAll.ijm” macros include: (1) a pre-processed image file that contains only the target channel, and for 3D images it has also been equalized for image intensity across the z-range; (2) a filtered image file that contains the Gaussian and Morphological Top-hat Filtered image from the pre-processed image; (3) an image (or image stack for 3D images) that contains center pixels (or connected voxels for 3D images) within the noise level corresponding to the median gray value of the middle z-slice) of identified dots at the selected threshold level; (4) an image (or image stack for 3D images) that contains enlarged dots by dilating from the center pixels (or connected voxels for 3D images) by the specified dot radius (the same parameters used by the morphological top-hat filter) to facilitate visualization; (5) (for “smFISH-thresholdOptimization.ijm” only) three diagnostic plots with the x-axis being the threshold level and the y-axis being the total dot number (can be normalized, linear or log-scale); (6) (for the entire input folder) a summary text file containing a tabulated summary of results; (7) (for the entire input folder) a “log_files” folder containing the running logs of each run, and a reference image for intensity normalization: for 2D, this is the first processed image; for 3D, this is the middle slice of the first processed image. Example output images and plots are shown in Figure 6.

REAGENTS AND SOLUTIONS

1. Organ Culture Media: DMEM/F12 supplemented with 150 µg/mL vitamin C and 50 µg/mL transferrin
 1. Starting Material:

Vitamin C (Sigma-Aldrich, A7506)

Ultrapure Water (e.g., Quality Biological 351-029-131)

Transferrin (Sigma-Aldrich, T8158)

DMEM/F-12 (Thermo Fisher, 11039021)

0.22 µm Steriflip filter (MilliporeSigma, SCGP00525)
 2. Make 75 mg/mL vitamin C stock solution (500×). Weigh 1500 mg of vitamin C in a 50 mL conical tube, and add 20 mL of Ultrapure water. Vortex the tube to mix, and pass the solution through a 0.22 µm filter to sterilize (use any sterile filtration system you have; for example, you can use a Steriflip filter). Pipet into 1 mL and 20 µL aliquots, and store them frozen at −20°C.

In practice, it is difficult to obtain an exact weight of powder; instead, it is much easier to calculate the volume of water required to bring a random weight to the correct concentration. For example, if you happen to weigh out 1280 mg vitamin C, just add $1280 / 75 = 17.07$ mL water for a final 75 mg/mL solution.
 3. Make 25 mg/mL of transferrin stock solution (500×). Weigh 25–100 mg of transferrin (Sigma-Aldrich T8158) in a sterile 15 mL conical tube, and add in 1–4 mL of Ultrapure water to bring transferrin into solution. Pipet into 1 mL and 20 µL aliquots, and store them frozen at −20°C.
 4. On the day of usage, thaw a 20 µL aliquot of both vitamin C and transferrin to make the Organ Culture Media. To make 1 mL, add 2 µL of vitamin C stock solution and 2 µL of transferrin stock solution into 1 mL of DMEM/F-12. Make fresh on the day of usage, and store at RT.
2. Fixative Solution: 4% paraformaldehyde (PFA) and 1× PBS in DEPC-treated water
 1. Starting Material:

16% PFA (Electron Microscopy Sciences, 15170)

10× PBS (e.g., Lonza, 51226; 0.017 M KH₂PO₄, 0.05 M Na₂HPO₄, 1.5M NaCl, pH 7.4)

DEPC-treated Water (e.g., Quality Biological, 351-068-131)

2. To make 1 mL of Fixative Solution, mix 650 μ L of DEPC-treated water, 100 μ L of 10 $\times$ PBS and 250 μ L of 16% PFA in a nuclease-free 1.5 mL tube. Make fresh on the day of usage, and store at RT.
3. PBSTx: 0.2% Triton-X-100 and 1 $\times$ PBS in DEPC-treated water
 1. Starting Material:

Triton-X-100 (Sigma-Aldrich, T9284)

Ultrapure Water (e.g., Quality Biological 351-029-131)

10 $\times$ PBS (e.g., Lonza, 51226; 0.017 M KH_2PO_4 , 0.05 M Na_2HPO_4 , 1.5M NaCl, pH 7.4)

DEPC-treated Water (e.g., Quality Biological 351-068-131)
 2. Make a 20 % Triton-X-100 stock solution in Ultrapure water (100 $\times$ 0.2%), because it is difficult to pipet 100% Triton-X-100 accurately due to its high viscosity and stickiness. In a marked 50 mL conical tube, pour in less than 10 mL of Triton-X-100. Fill in Ultrapure Water to the mark of 5 times of the Triton-X-100 volume. Invert the tube and shake vigorously to mix. Place the tube on a rotator for 1 hour to overnight, until the solution looks homogenous.
 3. To make 50 mL of PBSTx, mix 44.5 mL of DEPC-treated water, 5 mL of 10 $\times$ PBS and 500 μ L of 20% Triton-X-100 in a nuclease-free 50 mL tube. Store at RT.
4. PBS-SDS: 0.5% SDS and 1 $\times$ PBS in DEPC-treated water
 1. Starting Material:

20% SDS (e.g., Quality Biological, 351-066-721)

10 $\times$ PBS (e.g., Lonza, 51226; 0.017 M KH_2PO_4 , 0.05 M Na_2HPO_4 , 1.5M NaCl, pH 7.4)

DEPC-treated Water (e.g., Quality Biological, 351-068-131)
 2. To make 50 mL of PBS-SDS, mix 43.75 mL of DEPC-treated water, 5 mL of 10 $\times$ PBS and 1.25 mL of 20% SDS in a nuclease-free 50 mL tube.
5. Wash Solution: 2 $\times$ SSC and 10% formamide in DEPC-treated water
 1. Starting Material:

20 $\times$ SSC (e.g., K D Medical, RGF-3240; 0.3 M Sodium Citrate and 3 M Sodium Chloride)

Formamide (store at 4°C; Sigma-Aldrich, F9037)

DEPC-treated Water (e.g., Quality Biological 351-068-131)

2. To make 50 mL of Wash Solution, mix 40 mL of DEPC-treated water, 5 mL of 20× SSC and 5 mL of formamide in a nuclease-free 50 mL tube. Make fresh on the day of usage, and store at RT.
6. Hybridization Solution: 2× SSC, 10% formamide, 10% dextran sulfate and 50 µg/mL of yeast tRNAs in DEPC-treated water
 1. Starting Material:

20× SSC (e.g., K D Medical, RGF-3240; 0.3 M Sodium Citrate and 3 M Sodium Chloride)

Formamide (store at 4°C; Sigma-Aldrich, F9037)

50% Dextran Sulfate (MilliporeSigma, S4030)

10 mg/mL Yeast tRNAs (Sigma-Aldrich, R5636)

DEPC-treated Water (e.g., Quality Biological 351-068-131)

Positive Displacement Pipette (e.g., Gilson, M-1000R)

Pipette Tips for Positive Displacement Pipette (e.g., Gilson, CP1000ST)
 2. To make 10 mL of Hybridization Solution, mix 1 mL of 20× SSC, 1 mL of formamide, 2 mL of 50% dextran sulfate, 50 µL of 10 mg/mL yeast tRNAs and 6 mL of DEPC-treated water in a nuclease-free 50 mL tube. Vortex to thoroughly mix, and store frozen at −20°C.

The presence of 10% high molecular weight dextran sulfate (>500 kDa) accelerates the rate of hybridization by 10-fold. The 10% dextran sulfate stock solution is very sticky, so it is highly recommended to use a positive displacement pipette. A 50 mL tube is used to make the solution easier to vortex.

There is no need to aliquot, since this solution can be frozen and thawed more than 10 times.
7. DAPI Stock Solution: 50 µg/mL of DAPI in water
 1. Starting Material:

DAPI (Thermo Fisher, D1306)

Ultrapure Water (e.g., Quality Biological 351-029-131)
 2. Make 5 mg/mL concentrated DAPI stock. As this DAPI product comes as 10 mg of powder in an amber glass vial, just add 2 mL of water into the vial to produce a 5 mg/mL concentrated stock solution. Aliquot into 4 tubes of 500 µL, and take one to make a few 5 µL aliquots for convenient usage.
 3. Take one 5 µL aliquot of 5 mg/mL DAPI, add in 495 µL of water to obtain 50 µg/mL of DAPI in water. Store both the 5 mg/mL and the 50 µg/mL DAPI solutions frozen at −20°C.

8. 100 mM NaHCO₃, pH 8.30
 1. Starting Material:

Sodium Bicarbonate (Sigma-Aldrich, S5761)

Ultrapure Water (e.g., Quality Biological 351-029-131)

1 M HCl (for adjusting pH)

1 M NaOH (for adjusting pH)

Equipment:

Magnetic Stirrer

pH Probe

0.22 µm Steriflip filter (MilliporeSigma, SCGP00525)
 2. To make 50 mL of 100 mM NaHCO₃ (pH 8.30), weigh out 0.42 g of sodium bicarbonate. Put the powder in a 100–150 mL beaker. Add in 45 mL of ultrapure water to leave room for adjusting pH. Put in a clean stirrer bar and place the beaker on a magnetic stirrer. Use a pH probe to monitor the pH while stirring the solution. Adjust the pH by adding drops of 1 M HCl or 1 M NaOH until it reaches pH 8.30. Pour the solution into a 50 mL graduated tube, and add water to adjust the volume to 50 mL. Filter the solution through a 0.22 µm Steriflip filter (or use any other equivalent membrane filtration system you have). Store at RT.
9. 50% Glycerol in 1× PBS
 1. Starting Material:

Glycerol (e.g., Thermo Fisher, 15514011)

Ultrapure Water (e.g., Quality Biological 351-029-131)

10× PBS (e.g., Lonza, 51226; 0.017 M KH₂PO₄, 0.05 M Na₂HPO₄, 1.5M NaCl, pH 7.4)

Equipment:

Laboratory Rotator

(optional) 0.22 µm Steriflip filter (MilliporeSigma, SCGP00525)
 2. To make 50 mL of 50% Glycerol in 1× PBS, first pour glycerol (which will be viscous) to the 25 mL mark of a graduated 50 mL conical tube. Add 20 mL of ultrapure water and 5 mL of 10× PBS. Mix vigorously by shaking and inverting the tube. Rotate overnight at RT on an end-over-end laboratory rotator. The solution should be clear and homogenous after overnight mixing. You can optionally filter the solution through a 0.22 µm using a Steriflip filter, but it is not necessary if all components are high-quality reagents. Store at 4°C.

COMMENTARY

Background Information

smFISH provides an orthogonal approach to RNA sequencing-based techniques for gene expression analysis. While smFISH is not high-throughput in nature compared to sequencing-based techniques, it preserves the tissue organization context when analyzing gene expression. The described protocol in this unit is a basic form of smFISH, which has the advantages of high spatial resolution (single molecule), high-specificity (about 20 probes need to target the same molecule to be detected), and easy-to-perform (essentially only one-step hybridization). To demonstrate the high specificity of smFISH in mouse embryonic organs, I performed Cas9 smFISH using salivary glands from either the Cas9 transgenic (Platt et al., 2014) or wild type mice. Because Cas9 is a bacterial gene that is not present in mouse, I expect to see Cas9 smFISH dots only in the Cas9 transgenic but not wild type glands, which is indeed the case (Figure 1C). Nevertheless, the basic form of smFISH has the disadvantages of intrinsically low signal and low-throughput.

Many efforts have been made to improve either the signal intensity or the throughput of smFISH, or FISH in general. Signal amplification is often necessary to make FISH experiments practical in tissues with high autofluorescence background, such as mouse brain tissues. A hybridization chain reaction (HCR) has been used to amplify the signal of FISH experiments (Choi et al., 2010). A controlled HCR amplification has been used to greatly amplify the signal while preserving single-molecule resolution (schHCR; Shah et al., 2016). In addition, RNAscope (Wang et al., 2012) and PLISH (proximity ligation in situ hybridization; Nagendran et al., 2018) employ two different strategies to specifically amplify signals at the site where a pair of probes are in close proximity, thus preserving high specificity during signal amplification. On the other hand, increased throughput of single molecule or single cell FISH has been achieved either by sequential hybridization after DNase I-mediated probe removal (seqFISH; Lignell et al., 2017; Lubeck et al., 2014), or sequential channel barcoding following enzymatic removal of attached fluorescent dyes (MERFISH; Chen et al., 2015).

When analyzing gene expression in tissues, any of the above methods can be used depending on your research needs. These methods differ in their simplicity to perform and/or to analyze, reagent cost, signal intensity, resolution, specificity, and throughput. I prefer the basic form of smFISH for gene expression analysis in whole-mount mouse embryonic organs, because it has the highest possible resolution, and yet is relatively simple to perform and analyze.

Critical Parameters

As in all experiments examining RNA, it is critical to use RNase-free reagents and supplies to maximally avoid RNase contamination into samples. In addition, wear gloves throughout the protocol, and avoid talking when the solution and samples are exposed.

When designing probes, use as many probes as you can (the Stellaris Probe Designer allows up to 48 probes). In practice, at least 28 probes will generate robustly detectable smFISH dots. When the target RNA molecule is not long enough to allow for at least 20 probes,

consider alternative FISH techniques that amplify the signal, such as scHCR, RNAscope or PLISH.

When imaging smFISH samples, it is important to realize that smFISH signals are intrinsically low, and the dots are sub- or near-diffraction limit. Therefore, it is critical to adjust image acquisition parameters to make the pixel very small, use higher laser power than usual, and use line averaging to reduce the variation of background intensity (see *Basic Protocol 2*).

When quantifying smFISH images, the critical parameter is the user-selected threshold level. Manual selection of a threshold level inevitably involves a certain level of subjectivity that comes from the usual presence of ambiguous dim dots in smFISH images. The dim dots can originate from a low number of bound probes due to several possible causes: (1) partially degraded true target RNAs (either through physiological turnover or by contaminating RNases during sample processing); (2) full-length but difficult-to-access true target RNAs (e.g., tightly bound by proteins or other RNAs); and (3) non-specific RNAs. For 2D confocal images, dim dots can also result from visualization of slightly out-of-focus RNAs outside of the confocal slice that are otherwise perfectly probed. However, for smFISH images with high signal-to-noise ratio, the partial plateau on the “dot number vs. threshold level” plot curve (as shown in Figures 4C and 6B-C) should be obvious, so that a relatively objective threshold can be chosen near the mid-point of the partial plateau. Importantly, changing the threshold setting within this region does not significantly change the number of identified dots, so it is not necessary to be paranoid about the precise threshold choice. Importantly, however, the same threshold level should always be used for all images in the same experiment in the final analysis step (e.g., by “smFISH-processingAll.ijm”) to allow direct comparisons.

Troubleshooting

1. Autofluorescence

Tissues tend to have higher autofluorescence background than cultured cells. I have observed prominent individual autofluorescent cells residing in the mesenchyme of all examined mouse embryonic organs including salivary gland, lung and kidney (Figure 7). Therefore, when you begin doing smFISH experiments in a new type of tissue sample, you should always include a few unprobed control samples, and image them using identical acquisition parameters as the smFISH-probed samples to determine the autofluorescence pattern of your tissue.

2. Precipitation in Hybridization Solution

If you dehydrate and rehydrate your samples in the same 24-well plate used for probe hybridization, and do not remove the methanol-containing solutions from the plate, you may observe a white sticky precipitate in the Hybridization Solution the next morning. This is because the methanol has evaporated and contaminated the Hybridization Solution. In the presence of salt and sufficient methanol, the high molecular weight dextran sulfate in the Hybridization

Solution precipitates. To prevent this from happening, remove the methanol-containing solutions from wells of the 24-well plate you will use for probe hybridization.

Statistical Analyses

Understanding Results—Each clear dot on a smFISH image represents one target RNA molecule. Because at least 20 probes need to hybridize with the same molecule to generate a robust fluorescent dot, the detection specificity is ensured by the extremely low probability of multiple probes bind to the same non-specific target. As a result, the local smFISH dot density is a very good measurement of the local RNA expression level.

Importantly, smFISH does not rely on enzymatic signal amplification, so it is free of over-amplification artifacts. As a result, it is unnecessary to include negative controls using sense sequences or scrambled sequences, which is the common practice of conventional in situ hybridization. On the other hand, it is good practice to perform smFISH for mRNAs that are known to express in your tissue (e.g., *Cdh1* for epithelial organs), and exogenous mRNAs that do not express (e.g., bacterial gene *Cas9* for mouse organs), in order to know how positive smFISH dots and background signal look in your tissue of interest.

Time Considerations—Sample processing from dissection to sample mounting takes 2–4 days, depending on whether no co-immunostaining, 1-step co-immunostaining (*Alternate Protocol 1*) or 2-step co-immunostaining is performed. The total hands-on time is 5 to 8 hours spanning 4 days for a typical smFISH procedure with 2-step co-immunostaining (Figure 2A).

Imaging time can vary dramatically depending on whether a single z-slice or a z-stack image is acquired. Using the Nikon A1R system, acquiring one single-z image with 4-channels (3 passes) at 1024×1024 frame size with 16× line average takes 67 seconds. Imaging time will scale linearly as the number of stage positions, z-slices, channel series, frame size, and line average. Nevertheless, most modern microscope systems allow the user to set up a list of stage positions, so the hands-on time for imaging set-up is usually less than an hour.

Image analysis time depends on the number and size of images to analyze, as well as the hardware of your computer. For example, analyzing 3 of the supplied 2D test images (single z, 1024×1024; each smFISH channel is ~2 MB) using “smFISH-thresholdOptimization.ijm” on a MacBook Pro (2.9 GHz Intel Core i7 processor, 16 GB RAM) takes 70 seconds of hands-free processing time and about 2 minutes of user-involved threshold optimization. Analyzing all 12 test images using “smFISH-processAll.ijm” with a single selected threshold takes only 12 seconds in total (1 second for each image of ~2 MB).

Analysis of 3D images takes more time. For example, analyzing the 3 supplied 3D test images (50 z-slices, 399×399; this set of images was cropped to save testing time) using “smFISH-thresholdOptimization.ijm” on a MacBook Pro (2.9 GHz Intel Core i7 processor, 16 GB RAM) takes ~4 minutes of hands-free processing time, and 4 more minutes of user-involved threshold optimization. Once a threshold is chosen, it takes 76 seconds to analyze all 3 image stacks (~25 seconds for each image stack of ~16 MB).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance Statement

When studying gene expression in complex tissues like developing organs and tumors, it is usually not sufficient to determine only the global expression level of a particular RNA due to possible heterogeneous expression in different cells. Although single-cell RNA sequencing (scRNA-seq) has partly addressed this concern, the cell dissociation procedure required for scRNA-seq will inevitably destroy the spatial context of cells. To this end, single molecule RNA fluorescence in situ hybridization (smFISH) provides important complementary approaches by providing single-cell and even subcellular spatial characterization of gene expression in the context of tissue organization.

Figure 1. smFISH enables high-resolution gene expression analysis in mouse embryonic organs. (A) Schematic illustrating the principle of smFISH. Briefly, an array of 30 to 50 oligonucleotide probes is used to hybridize along the same RNA target. Each probe is 18–22 nucleotides in length and labeled with a single fluorescent dye. (B) Fluorescence confocal images of Cdh1 smFISH in mouse embryonic salivary gland, lung and kidney; single, discrete white dots above the diffuse white background identify individual RNA molecules. Note that the epithelium of lung and kidney has a single cell layer surrounding a cavity, whereas the salivary gland epithelium is filled with DAPI-labeled cells (blue) at this stage. (C) Fluorescence confocal images of Cas9 smFISH co-immunostained with anti-collagen IV (basement membrane marker) and anti-E-cadherin (epithelial junction marker) in E13 salivary glands from either Cas9 transgenic (top panels) or wild type mouse (bottom panels). Cas9 is of bacterial origin and is not expressed in wild type mouse. Thus, smFISH fluorescent dots are only observed in Cas9 transgenic but not wild type salivary glands,

demonstrating the specificity of smFISH. **(D)** Left: 3D reconstruction of fluorescence confocal image z-stack of Cas9 smFISH co-immunostained with anti-collagen IV and anti-E-cadherin in an E13 Cas9 transgenic salivary gland. Right: Cas9 smFISH channel overlaid with segmented spots in red. The 3D rendering and spot segmentation were performed with Bitplane Imaris software. Please note that the elongated appearance of 3D smFISH dots is due to the intrinsic property of light microscopy. In any light microscopy systems, the image of a point light source (known as the point spread function) is always elongated along the axial direction (commonly referred to as the z-direction). In practice, its size along the z-direction is often 3–5 times of that along the x-y directions. The red segmented spots are set to be spheres at the center of identified dots. Scale bars in all images are 10 μm .

A Procedure of smFISH in Mouse Embryonic Organs

B Making Bent-tip Forceps

C Mounting Samples in Curing Mountant

Figure 2. Overview of the smFISH workflow.

(A) Schematics illustrating the step-by-step procedures of smFISH followed by a 2-step co-immunostaining. (B) Schematic demonstrating how the bent-tip forceps are made. (C) Schematic demonstrating the mounting procedure using curing mounting media.

A Terminal Transferase-Mediated smFISH Probe Labeling **Figure 3. Enzymatic labeling of smFISH probes.**

(A) Schematics illustrating the major steps to produce and purify smFISH probes from unlabeled DNA oligonucleotides. (B) Fluorescence confocal images of Cdh1 smFISH in E13 salivary glands using commercial or custom-labeled probes. Top panels are merged images of smFISH and DAPI channels, whereas bottom panels are the smFISH channels in gray scale. Scale bar, 10 μm .

Figure 4. Semi-automated image analysis using ImageJ macros.

(A) Configuration of the Bio-Formats plugin in Fiji to enable support for selected image formats by checking “Enabled” and to suppress the “opening” dialog by checking “windowless” when the corresponding format is highlighted (Nikon ND2 in this example). (B) Interface to enable download and installation of MorphoLibJ plugin by enabling the “IJPB-plugins” update site. (C) Step-by-step instructions of the “smFISH-thresholdOptimization.ijm” macro user interface. Magenta color indicates added explanatory information, whereas all other images are direct screenshots of a Mac computer screen.

Figure 5. Additional user interfaces of ImageJ macros.

(A) The parameter interface of the “smFISH-processAll.ijm” macro. (B) The user interface for inspecting results when the “Show results one-by-one after processing all” checkbox of the “smFISH-thresholdOptimization.ijm” or “smFISH-processAll.ijm” is checked. (C) The threshold optimization user interface when processing 3D images. Magenta color indicates added explanatory information; the images are direct screenshots of a Mac system computer screen.

Figure 6. Example output images and plots from the smFISH analysis macro.

(A) The original smFISH image (left panel), the filtered image (middle), and the corresponding enlarged dots (right) of a Cdh1 smFISH of E13 salivary gland. The magenta insets are magnified 3 times. (B) Plot of normalized dot number vs. threshold levels. The black line is plotted in linear scale, whereas the blue line is plotted in log scale to facilitate the inspection of near-0 values. Note that the plot curve has a kink point followed by a partial plateau, indicating where the identified dot number is relatively insensitive to the change of threshold. (C) On the log-scaled total dot number vs. threshold level plot, the manually chosen threshold (x-axis) and the corresponding total dot number (y-axis) are marked by the red cross. Note that the manual selection falls close to the mid-point of the partial plateau on the plot curve. (D) Montage of z-stack images of the original, filtered and

enlarged dots at indicated z-slice number from a Cas9 smFISH of E13 salivary gland isolated from a Cas9 transgenic mouse. Scale bars, 10 μm .

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Figure 7. Autofluorescent cells in the mesenchyme of mouse embryonic organs. (A-B) Fluorescence confocal images of E13 salivary glands either probed with Cdh1 smFISH probes (A) or unprobed (B). (C-D) Fluorescence confocal images of unprobed E12.5 lung (C) and kidney (D). Black asterisks mark the autofluorescent cells in the mesenchyme of these organs. White dashed lines mark the epithelium-mesenchyme boundaries. Nuclear staining by DAPI is in blue. Scale bars, 10 μ m.