Chapter 7

smFISH: Quantifying and Mapping RNA at the Single-Molecule Level

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Abstract

RNA localization regulates spatial and temporal control of protein synthesis, influencing processes such as development, neuronal signaling, cell migration, and stress responses. Single-molecule fluorescence *in situ* hybridization (smFISH) enables direct visualization and quantification of individual transcripts within cells, providing both absolute counts and subcellular localization of RNAs. Here, we present a detailed, step-by-step protocol for smFISH in adherent mammalian cells, including probe design and labeling, sample preparation, hybridization, imaging, and analysis. This guide offers a reproducible workflow that facilitates the study of RNA localization and expression at single-molecule resolution.

Keywords smFISH, RNA localization

1 Introduction

RNA localization can dictate gene expression and is a critical form of post-transcriptional gene regulation. Messenger RNAs (mRNAs) are synthesized in the nucleus and must be exported to the cytoplasm to translate into proteins. After exiting the nucleus, mRNAs can be further compartmentalized to regulate local translation and underlie many diverse biological processes. For example, in neuronal cells, mRNA targeting to axons and dendrites is critical for plasticity, learning, and responding to stress [1–4]. Further, many studies have demonstrated that mRNA localization is important for proper *Drosophila* development, best exemplified by *bicoid* and *oskar* RNAs that help establish polarity in the oocyte via local translation on anterior and posterior poles [5–12]. RNA localization also contributes to the cellular stress response, as constitutively expressed transcripts can be sequestered into stress granules, while translating stress-induced gene mRNAs are inhibited from localizing to stress granules [13, 14]. Misregulation of RNA localization is associated with human diseases, including neurodevelopmental disorders, neurodegeneration, and

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cancer [15–18]; thus, there is a critical need to have techniques to visualize RNA localization. Indeed, single-molecule fluorescence *in situ* hybridization (smFISH) enables the direct visualization and quantification of individual transcripts within cells, revealing both their subcellular positioning and abundance.

smFISH builds on early fluorescence *in situ* hybridization methods and was first described in the late 1990's as a way to visualize single transcripts using multiple short, fluorescently labeled DNA probes hybridized along a target RNA [19, 20]. This approach provides absolute transcript counts as well as precise spatial information within fixed cells and tissues. smFISH involves fixation and permeabilization of samples, probe hybridization, and probe detection with fluorescence microscopy (Fig. 1). Immunofluorescence or imaging of fluorescently labeled proteins in the same samples permits investigation of RNA colocalization with proteins and/or compartmentalization in different cell types or states. Compared to bulk transcriptomic methods, smFISH offers direct spatial resolution at the single-cell level, making it a cornerstone technique for dissecting RNA localization, cellular heterogeneity, and regulatory mechanisms of gene expression.

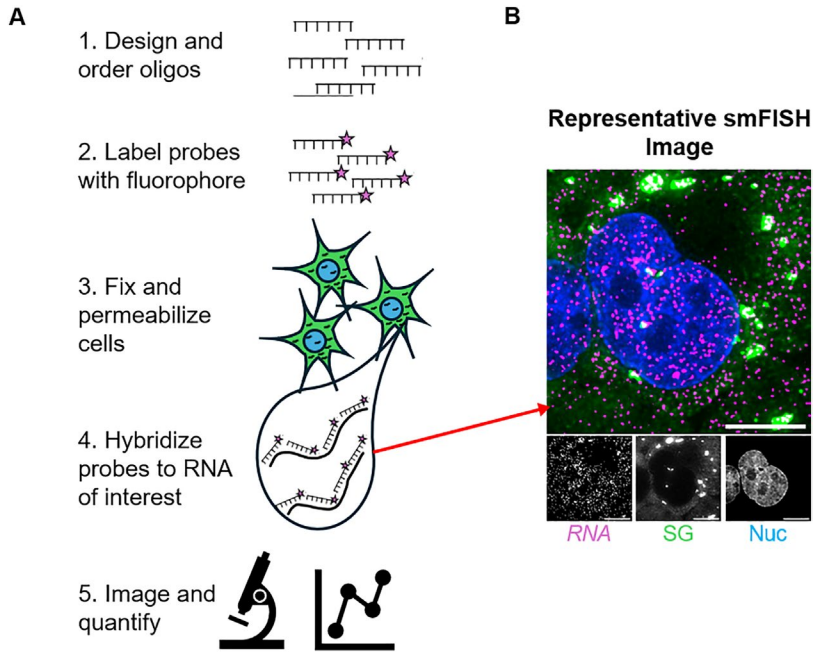

Fig. 1 Overview of single-molecule fluorescence *in situ* hybridization (smFISH) protocol. (**A**) Summary of step-by-step protocol to perform smFISH in adherent mammalian cells. (**B**) Representative smFISH image. U-2 OS cells were stressed for 45 min with 250 μM arsenite prior to fixation. Magenta represents RNA hybridized with FISH probes labeled with ATTO-647. Green is the stress granule (SG) marker, G3BP1, tagged with GFP, and the blue is nuclei stained with Hoechst #33342. Images are max intensity projections deconvolved using the Richardson-Lucy deconvolution. Scalebar = 10 μM

In this chapter, we provide a practical guide for performing smFISH in adherent mammalian cells, covering all stages from probe design and labeling to sample preparation, hybridization, and imaging. We detail experimental considerations for fixation and permeabilization that preserve RNA and cellular structure, as well as strategies for optimizing probe hybridization. Finally, we describe approaches for imaging and quantitative analysis, including methods for identifying and counting individual transcripts. Together, these procedures provide a reproducible workflow for visualizing and quantifying RNA localization at single-molecule resolution.

2 Materials

2.1 Probe Labeling

1. Oligonucleotides to RNA target.
2. Terminal deoxynucleotidyl Transferase (TdT) Enzyme and Buffer (Thermo Scientific).
3. ddUTP-dye (such as ddUTP-ATTO565, ddUTP-ATTO633, ddUTP-ATTO647N, or ddUTP-Cy5; Jena Biosciences).
4. RNase-free water.
5. Thermocycler.
6. Spectrophotometer.
7. PCR-strip tubes.

2.2 Performing smFISH on Adherent Mammalian Cells with Optional Sequential Immunofluorescence

1. Adherent tissue culture cells, such as U-2 OS cells.
2. Humidified cell culture incubator with 5% CO_2.
3. Cell culture media.
4. Phosphate-buffered saline (PBS).
5. 4% paraformaldehyde (PFA) solution diluted in PBS.
6. 96-well glass-bottom plates (#1.5H) or equivalent sterilized coverslip in a 12-well cell culture dish.
7. 12-well culture dishes (if using coverslips).
8. Wide-field fluorescence microscope with a high numerical aperture (>1.3) and a 60–100× oil-immersion objective.
9. 20× SSC:
   a. 175.3 g of NaCl (3 M).
   b. 88.2 g of sodium citrate (0.3 M).
   c. To 1 L with Molecular Grade H_2O.
10. Deionized formamide.
11. Wash Buffer A (WBA):
    a. 15 mL 20× nuclease-free SSC.
    b. 120 mL Molecular Grade H_2O.

12. Wash Buffer B (WBB):
    a. 15 mL 20× nuclease-free SSC.
    b. 135 mL nuclease-free water.
13. 0.1% Triton-× 100:
    a. 100 µL of 10% Triton-× 100.
    b. 9.9 mL of PBS.
14. RNase inhibitor (Ribolock).
15. Reagents for immunofluorescence (**Optional**).
    a. Primary and secondary antibodies.
    b. Bovine serum albumin (BSA).
    c. Antibody Dilution (Abdil) Buffer in PBS:
        i. 0.5% Triton-× 100.
        ii. 3% BSA.
    d. 3% BSA in PBS.
16. NucBlue with Hoechst #33342 (**Optional nuclear stain**).
17. Hybridization buffer
    a. 2 mL 50% dextran sulfate.
    b. 1 mL deionized formamide.
    c. 1 mL 20× nuclease-free SSC.
    d. 6 mL nuclease-free H_2O.

2.3 Software for Analysis

1. Python V3.6 or greater with BigFISH [21].
2. ImageJ/FIJI.

3 Methods

3.1 Probe Design

Properly designing probes is essential for the specificity of smFISH. Ensure you are using the primary transcript of interest (keeping in mind splicing isoforms), use Stellaris's designer to match the organism of interest, and leave masking level as high as possible to reduce potential off-target hybridization. Increasing the number of probes will increase the signal-to-noise ratio of your smFISH spots. Following the recommended spacing and probe size is also critical for specificity and proper hybridization.

1. Design DNA probes using Stellaris Probe Designer: https://oligos.biosearchtech.com/stellaris-designer
2. Leave the masking level at 5. Lower masking levels increase the chances of non-specific binding.
3. Design 25–48 probes for RNA target.

4. Select probe length of 20 nt with 2 nt spacing.
5. Insert your target sequence and hit Design Probes.
6. Order the probes (e.g., from IDT) either desiccated or resuspended in RNase-free water at 200 μM concentration.
7. Make a master mix of probes by adding 5 μL of each probe to a tube.

3.2 Probe Labeling and Clean Up

This protocol was adapted from [22, 23], which provides a more detailed explanation of the labeling reaction.

1. To make the labeling reaction, mix the following reagents in a PCR strip tube
   a. 5 μL of oligonucleotide probe master mix.
   b. 2 μL of ddUTP-fluorophore (1 mM).
   c. 3 μL of 5× TdT buffer.
   d. 1 μL TdT (30 U).
   e. 4 μL nuclease-free water.
2. Incubate reaction in a thermocycler with a heated lid for 20–24 h at 37°C.
3. Adjust the volume of reaction to 50 μL with nuclease-free water.
4. Use Zymogen's Oligo cClean and concentrate kit to clean up after probe labeling and elute in 25 μL (*see* **Note 1**).
5. Measure the absorbance of labeled probes at 260 nm and the wavelength of dye used (*see* **Note 2**) using a spectrophotometer. Calculate probe concentration and labeling efficiency using the following equations:

$$[DNA] = \frac{(A_{260} \cdot 33) \cdot 0.001}{(L \cdot 308.97) + 79.97} \cdot 10^6$$

$$[Fluor] = \frac{A_{Fluor}}{\varepsilon \cdot b} \cdot 10^6$$

$$Labeling\ Efficiency = \frac{[Fluor]}{[DNA]}$$

Here, A260 is the concentration absorbance of DNA at 260 nm multiplied by 33 to obtain ng/μL of DNA. This number is divided by the nucleotide length (L) multiplied by the average nucleotide molecular weight plus the terminal phosphate to calculate DNA molarity (μM). The fluorophore

concentration in μM is calculated using Beer-Lambert's law using the extinction coefficient of the fluorophore (ε) and a path length of 1 centimeter (could vary depending on the spectrophotometer being used). The labeling efficiency is determined by dividing the concentration of labeled probes (Fluor) by the total DNA concentration.

6. If labeling efficiency is below 70%, repeat steps 1 to 5, replacing oligonucleotide probe master mix with your eluate from step 4.
7. Dilute probes to 12.5 μM in nuclease-free water and store protected from light at −20°C.

3.3 Performing smFISH on Adherent Mammalian Cells (with Sequential Immunofluorescence)

All reagents and solutions should be sterile, and when possible, RNase-free.

1. Plate adherent cells in a 96-well glass-bottom plate or on top of a sterilized coverslip in a 12-well cell culture dish such that they reach ~70%–80% confluency by the time of the experiment (*see* **Notes 3 and 4**).
2. After your experiment, wash the cells by adding 200 μL (96-well plate) or 250 μL (12-well dish) of PBS to each well.
3. Remove the PBS. In a fume hood, add 200 μL (96-well plate) or 250 μL (12-well dish) of 4% PFA to fix the cells (*see* **Note 5**).
4. Incubate at room temperature for 10 min.
5. Remove PFA into the appropriate waste container.
6. Wash two times with 200 μL (96-well plate) or 250 μL (12-well dish) of PBS.
7. **DECISION POINT**: If performing immunofluorescence, follow these next few steps. If no immunofluorescence is necessary for your samples (e.g., GFP-tagged cell lines), move on to permeabilization with Triton at step 8 (*see* **Note 6**).
    a. Permeabilize in 100 μL of Abdil Buffer + 0.4 μL Ribolock (96-well plate) or 250 μL Abdil Buffer + 1 uL (12-well dish) for 10 min at room temperature (*see* **Note 7**).
    b. Wash once in 200 μL (96-well plate) or 250 μL (12-well dish) PBS.
    c. Incubate for 1 hour with primary antibody of interest in 100 μL 3% BSA + 0.4 μL of Ribolock (96-well plate) or 250 μL of 3% BSA + 1 μL of Ribolock (12-well dish).
    d. Wash three times in 200 μL (96-well plate) or 250 μL (12-well dish) PBS.
    e. Incubate for 1 h with secondary antibody of interest in 100 μL of 3% BSA + 0.4 μL of Ribolock (96-well plate) or 250 μL 3% BSA + 1 μL Ribolock (12-well dish).

f. Wash three times in 200 µL (96-well plate) or 250 µL (12-well dish) PBS.

g. Fix cells again with 200 µL (96-well plate) or 250 µL (12-well dish) of 4% PFA for 10 min at room temperature.

h. Wash two more times in 200 µL (96-well plate) or 250 µL (12-well dish) PBS, and continue to WBA wash step (Step 10).

8. To permeabilize cells if no immunofluorescence is necessary, incubate cells with 100 µL of Triton-X 100 (0.1%) + 0.4 µL of RNase inhibitor (Ribolock) (96-well plate) or 500 µL 0.1% Triton-X 100 + 2 µL Ribolock (12-well dish).
9. Incubate at room temperature for 5 min.
10. To wash, incubate with 200 µL (96-well plate) or 1 mL (12-well dish) WBA + 10% formamide for 5 min at room temperature.
11. Remove WBA into a designated waste container, as formamide is toxic.
12. Hybridize smFISH probes to RNA of interest by adding 75 µL (96-well plate) or 100 µL (12-well dish) of hybridization buffer + 1 µL of purified probes (12.5 µM) to each well/coverslip. If working with coverslips, flip the cell-side down onto a droplet of hybridization solution on parafilm over wet paper towels in a petri dish.
13. Wrap the 96-well plate or petri dish in parafilm and incubate at 37°C for 16–20 h protected from light.
14. Wash once in 200 µL (96-well plate) or 1 mL (for coverslips placed into a new 12-well dish) of WBA + 10% formamide + NucBlue dye (1 drop of NucBlue for 1 mL of WBA; *see* **Note 8**). If working with a coverslip, remove the coverslip from the incubation chamber into a 12-well dish.
15. Incubate for 30 min at 37 °C protected from light.
16. Remove WBA and wash once more in 200 µL (96-well plate) or 1 mL (12-well dish) WBA + 10% formamide.
17. Incubate for 30 min at 37°C protected from light.
18. Remove WBA, add 200 µL (96-well plate) or 1 mL (12-well dish) of WBB, and continue to imaging (*see* **Note 9**).
19. If working with coverslips, mount the coverslip onto a slide with the cell-side down and seal with clear nail polish.

3.4 Image Acquisition and Analysis

Optimized imaging parameters and proper analysis tools are essential for high-quality smFISH data. We recommend creating a custom pipeline depending on your use case with BigFISH/FISH-Quant functions built in to reduce bias and increase reproducibility. These use cases could include subcellular localization of the RNA, cellular heterogeneity, or total RNA counts.

1. To acquire high-resolution images of single FISH spots, we recommend acquiring images in either widefield or HILO (Highly Inclined Thin Illumination) on a widefield microscope with a 100X oil objective.
2. Determine imaging parameters optimal for your experiment (e.g., exposure time and laser intensity).
3. Next, determine the Z slices necessary to acquire the full cell volume, 13–15 Z-slices at 0.2 μm intervals for U-2 OS cells are sufficient, if necessary.
4. Collect three images in each well for analysis.
5. For image analysis, we highly recommend using BigFISH/FISH-Quant pipeline to accurately count single-molecule FISH spots, segment cells and nuclei, and determine subcellular localization (*see* **Note 10**).
    a. Import raw images into the BigFISH pipeline and recommend segmentation of cells and nuclei using a pretrained U-net model available from BigFISH using TensorFlow.
    b. Select well-segmented cells and perform downstream smFISH analysis within those cells.
    c. Identify threshold values for smFISH spots that represent your images. FISH spots are distinct single points and should have enough contrast from the background to be visualized by eye to ensure proper spot counting. We highly recommend hand-counting FISH spots with ImageJ's Cell Counter function when establishing a new pipeline for smFISH analysis.
6. After images are unbiasedly analyzed, select the image or crop of the image in ImageJ/FIJI such that it matches your quantifications (*see* **Note 11**).

4 Notes

1. TdT buffer is toxic and should be disposed of in a proper waste container.
2. Max absorption and extinction coefficient will depend on the fluorophore being used. For example, absorbance ATTO647N would be measured at 646 nm and an extinction coefficient of 1.5×10^5 M^{-1} cm^{-1}. We recommend checking the absorbance max and extinction coefficient on FPbase.
3. Adjust cell confluency as you would for other experiments. Overly confluent cells can cause cells to overlap with each other, reducing the single-cell power of smFISH.

4. A glass-bottom dish or coverslip (#1.5 H) is necessary for smFISH to ensure high-resolution images on 100×/1.4 NA oil objectives.
5. Other fixatives, such as methanol, can be used.
6. Using a cytoplasmic marker will be critical for automated downstream analysis of cytoplasmic RNAs. We routinely use the stress granule marker G3BP1 tagged with GFP to define the cytoplasm. Using a cytoplasmic marker will ensure proper segmentation of cells to garner single-cell information. Ensure the fluorophore used for your cytoplasmic marker does not spectrally overlap with your smFISH probes by checking FPbase.
7. RNase inhibitors are essential to add to all permeabilization or blocking steps to limit RNA degradation.
8. Similarly, to Note 6, a nuclear stain is necessary for downstream analysis for subcellular and single-cell localization of smFISH spots with BigFISH/FISH-Quant. We routinely use NucBlue with Hoechst #33342 for nuclei staining.
9. We typically image immediately or 1–3 days after final wash (with plates stored at 4°C). Longer-term storage can reduce fluorescence signal and image quality. This can be overcome by other preservation techniques.
10. We highly encourage researchers to use the BigFISH pipeline to analyze smFISH images. It encompasses segmentation, spot counting, cluster analysis, and subcellular single-cell localization features. Further, the documentation is clear and robust for performing analysis in Python.
11. We typically deconvolve our representative images first on Nikon Elements using a Richardson-Lucy Deconvolution algorithm.

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