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Shinichi Nakagawa
Tetsuro Hirose *Editors*

Nuclear Bodies and Noncoding RNAs

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Nuclear Bodies and Noncoding RNAs

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Preface

In the nucleus of higher eukaryotes, proteins and nucleic acids are nonrandomly distributed and constitute distinct nuclear bodies that control specific nuclear processes such as biogenesis of ribosomes, regulation of gene expression, pre-mRNA splicing, and modification and assembly of ribonucleoprotein complexes. The compartmentalized organization of the nucleus is considered to provide one of the cellular bases for the sophisticated regulation of gene expression found in the higher eukaryotes. Interestingly, recent studies revealed that certain long non-protein-coding RNAs accumulate in specific nuclear bodies and regulate the function of the nuclear bodies by serving as architectural components or controlling the localization or dynamics of associating protein components. This book focuses on cytological, biochemical, and molecular biological methods to identify and examine the function of each nuclear body, with an emphasis on the analysis of long noncoding RNAs.

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Part I

Imaging of Noncoding RNAs and Nuclear Bodies

Chapter 1

Visualization of lncRNA by Single-Molecule Fluorescence In Situ Hybridization

Margaret Dunagin, Moran N. Cabili, John Rinn, and Arjun Raj

Abstract

Single-molecule RNA fluorescence in situ hybridization is a technique that holds great potential for the study of long noncoding RNA. It enables quantification and spatial resolution of single RNA molecules within cells via hybridization of multiple, labeled nucleic acid probes to a target RNA. It has recently become apparent that single-molecule RNA FISH probes targeting noncoding RNA are more prone to off-target binding yielding spurious results than when targeting mRNA. Here we present a protocol for the application of single-molecule RNA FISH to the study of noncoding RNA as well as an experimental procedure for validating legitimate signals.

Key words Single-molecule RNA FISH, lncRNA, Noncoding RNA, Fluorescence in situ hybridization, Single cell

1 Introduction

Many recent experiments have revealed that the genome is filled with a large number of long RNA molecules that do not encode proteins [1]. These long noncoding RNAs (lncRNAs) have been associated with a large number of cellular processes, including differentiation and disease onset and progression [2–4]. Yet, many fundamental characteristics of lncRNAs remain unclear, even as basic as absolute abundance and subcellular localization. These arise from fundamental limitations of biochemical assays.

RNA fluorescence in situ hybridization (RNA FISH) is a technique that in many ways provides a useful complement to biochemical assays by labeling RNA molecules directly in the cell through hybridization of labeled nucleic acid probes to target RNA [5, 6]. Previous studies of lncRNA such as XIST, NEAT1, MALAT1, and Gomafu [7–13] have successfully deployed RNA FISH to visualize the localization of those molecules, in the case of XIST, showing that it localizes specifically to the inactive X chromosome [7, 8]. However, these studies targeted relatively abundant

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noncoding RNAs, and in general the sensitivity of traditional RNA FISH is too low to successfully visualize the majority of lncRNAs, which typically are relatively low abundance.

Single-molecule RNA FISH based on pools of short, labeled DNA oligonucleotides [14, 15] has the ability to detect individual molecules of RNA in situ, thereby affording it the sensitivity to detect even low-abundance lncRNA. Moreover, it is quantitative, enabling absolute quantification of the number and location of each target molecule within the cell. Researchers have used single-molecule RNA FISH to detect lncRNA with success [16–23], and in two cases, it has proven essential to deciphering the mechanism of action [18, 21]. However, in a recent systematic study of the application of single-molecule RNA FISH to lncRNA (Cabili et al. in submission), we found that lncRNAs can present unique challenges in terms of distinguishing nonspecific background from valid signals. The issue is that a single oligonucleotide in the pool may bind to a high-abundance, highly localized off-target within the cell, typically within the nucleus. Because of the high degree of sequence contamination with repetitive elements, lncRNA is more prone to these "rogue" oligonucleotides, and in contrast with mRNA, legitimate lncRNA signal often appears as bright blobs in the nucleus, making it more difficult to discount such signals as nonspecific background a priori.

We here present a protocol for single-molecule RNA FISH as applied to the detection of lncRNAs. The details of the protocol are similar to those in previous protocols we have published ([24], website: https://sites.google.com/site/singlemoleculernafish/), but we here also describe a means to distinguish legitimate signal from illegitimate signal. To do so, we label every other oligonucleotide with a differently colored fluorophore (i.e., odds labeled "red," evens labeled "green") and look for colocalization of the signal. If the signals colocalize, the signal is legitimate and unlikely due to a single oligonucleotide binding off-target, but if they do not colocalize, then it is possible that at least some of the observed signals are due to off-target binding. We describe the RNA FISH procedure, image acquisition, and elements of the image analysis involved in this approach.

2 Materials

2.1 Probe Design

We recommend designing and ordering single-molecule RNA FISH probes using the design tool available online at http://www.biosearchtech.com/stellarisdesigner/. The two-color colocalization technique for validating lncRNA probe sets requires three probe sets targeting the sequence of interest: a "whole probe" set comprised of all the oligos and then two subsets for validation purposes consisting of every other oligo in the whole probe set.

Any fluorophore is fine for the "whole probe" set; Quasar 570 (Cy3 equivalent) is a good choice. These "odds and evens" probe sets should be conjugated to two differently colored fluorophores (we recommend Quasar 570 and Quasar 670, which are Cy3 and Cy5 equivalents) so that you can image them together in the same sample. The total number of oligonucleotides required for robust visualization can depend on the details of the RNA target, but we have found that 20 oligonucleotides in the "whole probe" set is typically sufficient to produce quantifiable spots while still providing enough signal in the odds and evens probe sets to validate the signal. It is also important to validate the signal in every cell type under examination, because off-target signal can sometimes be cell type specific.

We recommend also targeting an mRNA control in a third color, e.g., fluorescein. One can target GAPDH mRNA or Cyclin A2 [25], which has the advantage of expressing in cells in the S, G2, and M phases of the cell cycle, enabling cross-correlation with cell cycle.

2.2 Solutions

1. 1× PBS: 5 mL 10× nuclease-free PBS, 45 mL nuclease-free water. Store at room temperature. Good for years.
2. Fixation buffer: 5 mL 10× nuclease-free PBS, 5 mL 37 % formaldehyde (formalin), 40 mL nuclease-free water. Store at room temperature, good for months. Use in a fume hood.
3. Wash buffer: 5 mL 20× nuclease-free SSC, 5 mL deionized formamide, 40 mL nuclease-free water. Allow formamide to warm to room temperature (store at 4 °C) before opening bottle to minimize oxidation. Store wash buffer at room temperature, good for months. Use in a chemical fume hood.
4. 70 % ethanol: 35 mL 100 % ethanol, 15 mL nuclease-free water. Store at room temperature. Good for months.
5. 2× SSC: 5 mL 20× SSC, 45 mL nuclease-free water. Store at room temperature. Good for years.
6. Hybridization buffer: 1 g dextran sulfate, 1 mL deionized formamide, 1 mL 20× nuclease-free SSC, 8 mL nuclease-free water. Add 1 g dextran sulfate to 6–7 mL of nuclease-free water and nutate tube slowly until dissolved. Add 1 mL 20× SSC and 1 mL room temperature formamide, and then add nuclease-free water to bring the total volume of the solution up to 10 mL. Make 500 µL aliquots and store at –20 °C. Frozen aliquots will last for years.
7. Anti-fade buffer: 850 µL nuclease-free water, 100 µL 20× SSC, 40 µL 10 % glucose (w/v), 10 µL 1 M Tris pH 8. Good for up to a week.
8. Glucose oxidase solution: 100 µL anti-fade buffer, 1 µL well-vortexed catalase, 1 µL glucose oxidase stock. Make fresh each time.

9. Glucose oxidase stock solution: 37 mg glucose oxidase, 10 mL 50 mM sodium acetate (pH ~5). Make aliquots and freeze at −20 °C. Each aliquot will be good for 10–20 freeze thaws, thaw right before use then return aliquot to freezer.
10. 1:100 DAPI working dilution: 1 μL DAPI (5 mg/mL), 99 μL nuclease-free water.
11. Probe stock solution: resuspend dried oligos in 400 μL of TE buffer to make a 12.5 μM stock.

2.3 Supplies

1. #1 2-Well chambered cover glass (e.g., Lab-Tek).
2. #1 Cover slips 18 × 18 mm.
3. Parafilm.
4. Kimwipes.
5. 10 cm tissue culture dish (other similar container).
6. Rubber cement (e.g., Elmers).
7. Fine point forceps.
8. Pipettes and pipette tips.
9. Microcentrifuge tubes.
10. Glass slides.

2.4 Microscopy Equipment

1. Standard widefield fluorescence microscope (e.g., Nikon TE2000/Ti, Zeiss Axiovert).
2. Strong light source, such as a mercury or metal-halide lamp (e.g., ExFo Excite, Prior Lumen 200). We have found that the metal-halide lamps are generally brighter, especially for the far red dyes such as Cy5. Newer LED-based light sources may work as well.
3. Filter sets appropriate for the fluorophores chosen (e.g., Chroma 41002 for Quasar 570, Chroma SP104v2 for Quasar 670).
4. Standard cooled CCD camera, ideally optimized for low-light level imaging rather than speed (13 mm pixel size or less is ideal; e.g., PIXIS 1024BR, Princeton Instruments, CoolSNAP HQ). We have found that EMCCDs do not provide any additional signal-to-noise benefits over more traditional cameras.
5. High NA (>1.3) 100× DIC objective (be sure to check transmission properties when using far red dyes such as Cy5 or Cy5.5). We have also seen spots using an oil-immersion 60× objective.

2.5 Chemicals

1. 10× Nuclease-free PBS.
2. Nuclease-free water.
3. 37 % formaldehyde (formalin).
4. 95 % ethanol.

5. Triton X-100.
6. Dextran sulfate sodium salt from *Leuconostoc* spp. avg MW >500,000.
7. 20× Nuclease-free SSC.
8. Deionized formamide.
9. DAPI, dilactate.
10. Glucose.
11. Catalase.
12. Glucose oxidase.
13. 1 M Tris pH 8.0.
14. 3 M Sodium acetate pH 5.2.
15. Tris-EDTA buffer pH 8.0.
16. 1× Dulbecco's PBS (DPBS).

3 Methods

3.1 Fixing Cells for RNA FISH

3.1.1 Cell Culture and Fixation of Adherent Cells

1. Grow adherent cells on 2-well, #1 chambered cover glass (*see* **Note 1**).
2. Aspirate growth media.
3. Wash each well with 1 mL 1× PBS (*see* **Note 2**). Aspirate.
4. Add 1 mL fixation buffer and incubate for 10 min at room temperature.
5. Aspirate fixative and wash twice with 1× PBS.
6. Add 1 mL 70 % ethanol to each well, parafilm 10 cm dish to prevent evaporation, and store at 4 °C at least overnight or for up to 3 months (*see* **Note 3**).

3.1.2 Cell Culture and Fixation of Suspension Cells

1. Centrifuge cultured cells in a 15 mL tube at 200 × *g* for 2 min (or at appropriate speed for cell type in question).
2. Aspirate growth media, leaving cells in a pellet at base of tube.
3. Gently resuspend cells in DPBS and centrifuge again.
4. Aspirate DPBS and resuspend cells in 50 μL of PBS (*see* **Note 4**).
5. Add 1–5 mL fixation buffer for 10 min at room temperature. Mix by pipetting or inverting the tube for the first 30 s of the 10 min incubation time.
6. Centrifuge to pellet cells and aspirate formaldehyde solution.
7. Add 1 mL 1× PBS and vortex gently to resuspend pellet. Centrifuge and aspirate. Repeat twice.
8. Resuspend cells in 2–3 mL 70 % ethanol.
9. Cap tube and store at 4 °C at least overnight or for up to 3 months.

3.2 Preparation of Probe for RNA FISH

1. Resuspend lyophilized probe in 400 μL of TE buffer to generate probe stock.
2. Prepare dilutions of probe stock in TE buffer. Make dilutions of 1:2, 1:10, and 1:20.
3. At first, try all dilutions as well as the stock concentration to empirically determine optimal concentration of probe.

3.2.1 Single-Molecule RNA FISH on Adherent Cells

1. Prepare hybridization solutions consisting of 50 μL hybridization buffer per well plus 1 μL of desired probe dilution (*see* **Note 5**). When performing single-molecule RNA FISH targeting lncRNA, one reaction should contain the full probe set and an mRNA control, and the other reaction should contain both the "odds and evens" probe sets and the mRNA control.
2. Remove cells from 4 °C and aspirate ethanol. If ethanol has evaporated, do not use cells.
3. Add 1 mL wash buffer to each well to equilibrate cells (*see* **Note 6**).
4. Apply 50 μL of hybridization solution to center of well, taking care not to disturb the cells with the pipette tip. Add an 18 × 18 mm cover slip to the top of the hybridization solution to spread the solution across the well and to minimize evaporation (*see* **Note 7**).
5. Form a wick by twisting and knotting a Kimwipe. Wet with approximately 700 μL of 2× SSC. The wick will keep the sample humidified during hybridization.
6. Place chambered cover glass and the wetted wick in a 10 cm dish and seal with parafilm (Fig. 1).

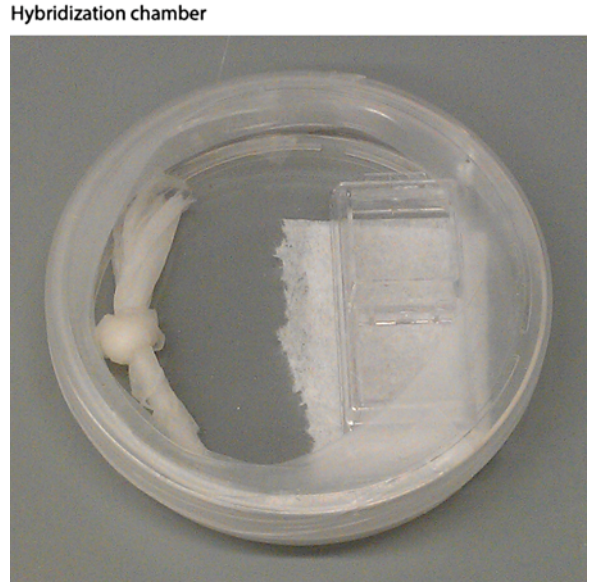

Fig. 1 A typical hybridization chamber set up with 2-well chambered cover glass and wetted wick

7. Place hybridization chamber at 37 °C overnight (*see* **Note 8**).
8. In the morning, add 1 mL wash buffer to each chamber and use hooked forceps to carefully remove the cover slip. Aspirate wash buffer.
9. Add 1 mL wash buffer to each well and incubate for 30 min at 37 °C.
10. Repeat wash with 1 mL wash buffer plus 1 μL of 1:100 DAPI at 37 °C for 30 min (*see* **Note 9**).
11. During the second wash, prepare 1 mL of anti-fade buffer per well. Transfer 100 μL per well of this to a microcentrifuge tube and add 1 μL each per well of vortexed catalase and glucose oxidase (*see* **Note 10**).
12. Aspirate wash buffer and add 1 mL 2× SSC.
13. Aspirate 2× SSC and add 900 μL of anti-fade buffer.
14. Aspirate anti-fade and apply 100 μL of glucose oxidase solution to each well. Cover with an 18×18 mm cover slip (*see* **Note 11**).

3.2.2 Single-Molecule RNA FISH on Suspension Cells

1. Prepare hybridization solution as above.
2. Invert tube with fixed cells several times to resuspend cells then place 50–500 μL of cells (depending on concentration) in a microcentrifuge tube (*see* **Note 12**).
3. Pellet cells and aspirate ethanol—there should be a small but visible pellet.
4. Gently resuspend cells in 500 μL of wash buffer with 0.1 % Triton X-100 (*see* **Note 13**).
5. Pellet cells and aspirate wash buffer.
6. Resuspend cells in 50 μL of hybridization solution plus probe. Mix well with gentle vortexing.
7. Incubate tube overnight at 37 °C.
8. Pellet cells and aspirate about 50 % of the hybridization buffer. The pellet is very fluffy and easy to lose at this point (*see* **Note 14**).
9. Add about 200 μL of wash buffer with 0.1 % Triton and pellet. Aspirate buffer, taking care not to disturb the pellet.
10. Add 500 μL wash buffer with 0.1 % Triton; resuspend and incubate at 37 °C for 30 min.
11. Centrifuge, aspirate supernatant, and add 500 μL wash buffer with 0.1 % Triton + 1 μL 1:100 DAPI; resuspend and incubate at 37 °C for 30 min.
12. Pellet cells, aspirate wash buffer, and wash twice with 500 μL 2× SSC plus 0.1 % Triton X-100.
13. Pellet cells, aspirate supernatant, and resuspend in 500 μL of anti-fade buffer.

14. Pellet cells, aspirate supernatant, and resuspend in about 30 μL of glucose oxidase solution (*see* **Note 15**).
15. Place 5 μL of cell suspension on a clean glass slide and cover with an 18 × 18 mm or 24 × 24 mm #1 cover slip (*see* **Note 16**).
16. Place a Kimwipe over the cover slip and apply gentle, even pressure over the surface of the cover slip to press it firmly onto the surface of the slide. Be careful to apply pressure straight down and not to allow the cover slip to slide horizontally which could result in sheared cells (*see* **Note 17**). The Kimwipe will wick up the excess liquid.
17. Seal around the edges of the cover slip with rubber cement and let dry in a dark place (*see* **Note 18**).

3.3 Imaging

1. Mount your cover slip on the microscope using immersion oil as appropriate (*see* **Note 19**).
2. Focus the microscope by looking at cells in the DAPI channel. Unless you have an unusually bright RNA FISH signal, such as XIST or MALAT1, the signal itself will not be visible through the eyepiece.
3. Take a picture in the mRNA control channel. We recommend exposure times of 2–3 s for most RNA FISH signals. You should see clear diffraction limited spots of uniform size (Fig. 2a). If you see clean signal in the mRNA channel, you can look at the lncRNA channel(s) to see what's there (Fig. 2b, c for examples of good signal and no signal) (*see* **Note 20**).
4. In the mRNA channel, navigate to the bottom of the cells by slowly lowering the plane of focus while taking images. You want to go slightly below the cells. Find the point where the cell is out of focus and the RNA spots are still visible but look out of focus, dim, and very diffuse.
5. Set up your imaging software to take a series of images at 0.3 μm spaced intervals, moving up through the cell in "slices." The number of planes to image depends on the thickness of the cells. Usually 25 planes for fibroblasts and 30 planes for HeLa or similar cells are a good place to start. Too few planes results in missing spots; excess focal planes can be removed manually after imaging. Set the software to take a "stack" of images in one color, save the images, then return to the starting z-position and take sequential stacks of images in all the required channels. The recommended order is Trans (take only five planes at 2 μm spacing), RNA FISH channels, and then DAPI (*see* **Note 21**).
6. When you are not actively imaging samples, store them at 4 °C. We image samples immediately after washing (i.e., the day after starting the hybridization), since the signal may start to fade over time.

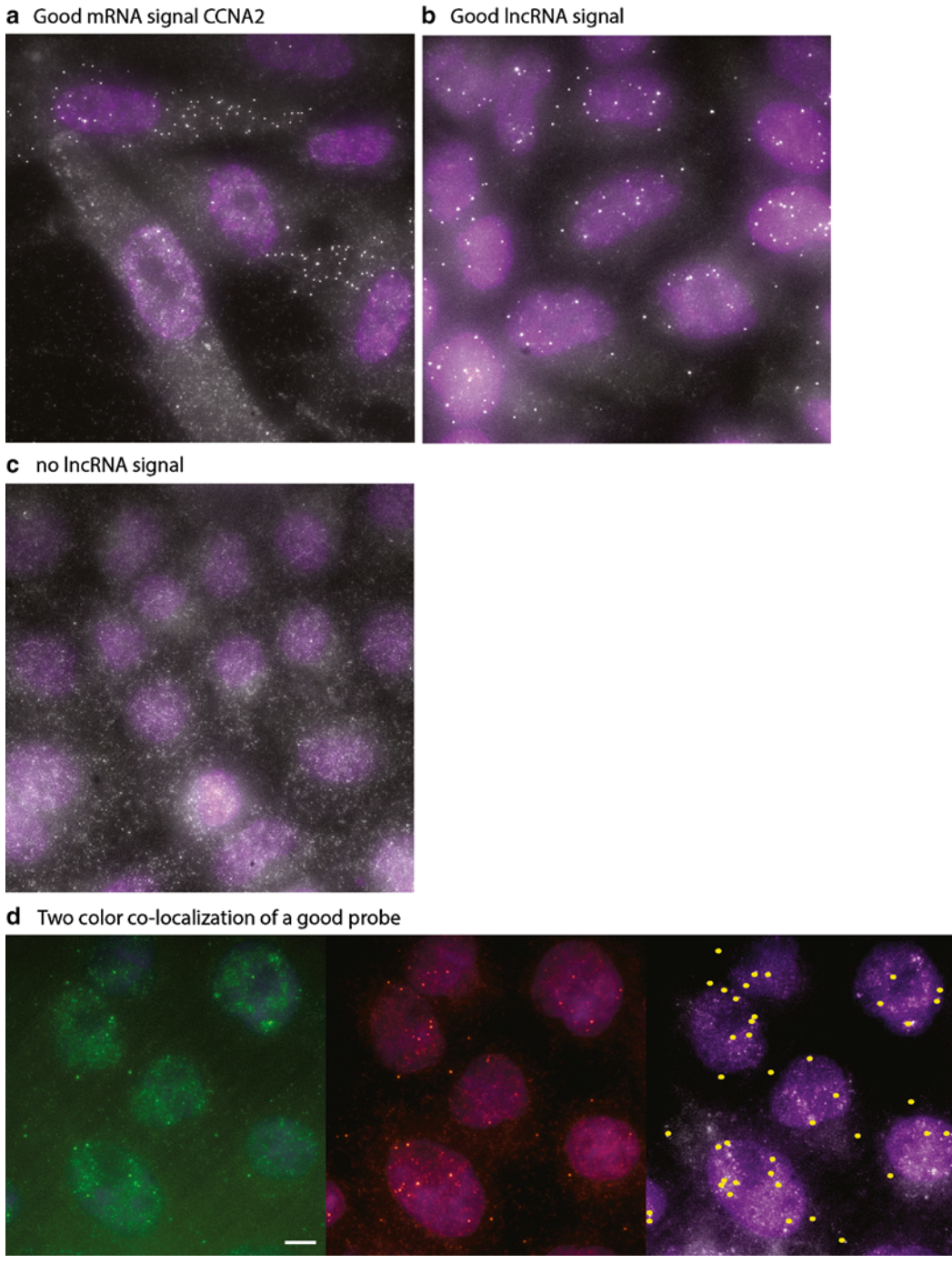

Fig. 2 (**a**) Representative example of CCNA2 mRNA signal in human lung fibroblast (HLF) in Cy5. (**b**) An example of lncRNA that has a clear signal (also valid by two-color colocalization, *see* **d**; Alexa 594). (**c**) Probing of a lncRNA that does not display any signal; the signal shown is attributed to background. (**d**) Demonstration of the two-color colocalization assay. Fluorescent micrographs of a lncRNA probe set in HeLa of the even-numbered oligonucleotides (*left*, *green*; Alexa 594), odd-numbered oligonucleotides (*middle*, *orange*; Cy3), and colocalized spots over the even-numbered set micrograph (*right*, *yellow over white*; Alexa 594). Scale bar, 5 μm (color figure online)

3.4 Analysis

1. To visually examine data, create a maximum z-projection of all of the slices in each stack (e.g., using ImageJ or MATLAB). This will make it easy to visualize all the spots in the 3D volume of the cell at once. If there are many out of focus planes either above or below the cells, you should remove them from the stack prior to creating the max merge.
2. Compare images taken in the odds and evens channels to each other to check whether spots in each channel colocalize by visual inspection. If you see good colocalization (Fig. 2d), you can use a computational approach to spot identification and localization to confirm that the number of colocalized signals is similar to those obtained from the "whole probe" signal and/or proceed to analyze the "whole probe" data (*see* **Notes 22**–**24**).
3. If the gene you are investigating is distributed throughout the nucleus and/or cytoplasm in distinct, punctate, single-molecule spots like those seen with mRNA FISH, quantify individual RNA FISH spots using the StarSearch software we have published online (rajlab.seas.upenn.edu/StarSearch/launch.html) (*see* **Note 25**).
4. Overlay images of RNA FISH channels with the DAPI channel to look at nuclear localization.

4 Notes

1. We find that 2-well chambered cover glasses (Lab-Tek) are ideal for ease of handling and for applying the two-color colocalization technique for validating lncRNA probe sets. We have found that using the Lab-Tek II chambered cover glasses can produce worse signals (they use #1.5 cover glass). You can use one well for the full probe set and the other for the split "odds and evens" probe sets. You can store chambered cover glasses in 10 cm tissue culture dishes. A small piece of Kimwipe should be placed under the chambered cover glasses to prevent them from sticking to the dish should they become wet.
2. We routinely use a variety of different media, and the type of media is not important in that it will not affect RNA FISH results. Use the media in which you normally culture your cells of choice. The most important point is to keep cells as healthy as possible. Stressed cells often have much higher levels of autofluorescent background which can interfere with imaging and obscure or mimic RNA FISH signal. We recommend fixing cells at about 70 % confluence.
3. Alternatively, we have grown cells on #1 cover slips (we have found that #1.5 cover slips can work sometimes, although

sometimes the spots are difficult to resolve). In this case, we typically place the cover slips in 6-well dishes or similarly sized container for growing the cells. It is also convenient to do some of the liquid handling in 6-well dishes, although you must take care to keep track of which side of the cover slip the cells are on. All subsequent steps of the protocol can be performed identically to cells grown in a chambered cover glass except where noted. The volume of buffers used might have to be adjusted slightly to make sure that the cells are completely covered by liquid at all times.

4. We use 70 % ethanol to permeabilize cells. While we generally leave the cells in ethanol at 4 °C at least overnight, if it is essential that you perform RNA FISH on cells the same day they are fixed, permeabilization will occur in 1 h in 70 % ethanol at room temperature. If ethanol levels in wells become low during storage, provided they have not dried out, more 70 % ethanol can be added to prevent them from drying. We recommend storing in 70 % ethanol for no longer than 3 months, although we have successfully performed RNA FISH on cells that have been stored in 70 % ethanol for over a year.
5. Resuspending cells in PBS helps prevent them from clumping together upon fixation. Large clumps of cells will be difficult to image and analyze.
6. We typically determine the ideal concentration of probe in hybridization solution empirically. In our experience, 1 μL of a 1:20 dilution of the stock solution of probe in 50 μL of hybridization buffer generally gives good signal. There is often a wide range of concentrations that will give a similar, quantifiable signal.
7. Do not allow the sample to dry between steps. Allowing the cells to dry, especially at any point after applying probe, can result in high levels of clumpy or spot-like background.
8. If cells were grown on cover slips, the hybridization solution can be placed in a drop on a piece of parafilm and the cover slip placed cell-side down on top of the drop. In this case, be careful that the wick does not come into contact with the hybridization solution. If using 4-well or 8-well chambered cover glass, adjust the amount of hybridization buffer accordingly and cover with an appropriately sized cover slip.
9. Hybridization can sometimes occur in as few as 4 h, but we usually leave it overnight, which allows for a full day of imaging after washes are completed the next morning.
10. One microliter of 1:100 DAPI almost always results in a good nuclear stain, but if the signal appears dim, more DAPI can be added to wells during imaging.

11. Anti-fade and glucose oxidase treatment are required to prevent photobleaching of Quasar dyes and Cy5. It is not necessary for oligonucleotides conjugated to dyes such as Alexa 594, Cy3, Atto 647N, Atto 700, and Atto 488. To mount cover slips on slides, 100 μL of glucose oxidase solution is enough for multiple samples. Note that glucose oxidase will lose its effectiveness over time when exposed to air at room temperature—for that reason, we will often store samples at 4 °C for later imaging.
12. To mount cells grown on cover slips, pipette a small volume of glucose oxidase solution (5–10 μL for an 18×18 mm or 24×24 mm cover slip, more for a bigger one) on a clean glass slide and place the cover slip in the bead of glucose oxidase solution. Use a Kimwipe to gently press down the cover slip and soak up any liquid that seeps out from under the cover slip. Seal around the edge of the cover slip with rubber cement.
13. Alternatively, you can fix cells in suspension and use poly-L-lysine or cytospin to adhere them to a cover slip after which you can perform RNA FISH following the protocol for adherent cells on a cover slip.
14. The use of Triton X-100 in the wash buffers helps to prevent cell loss due to cells sticking to the sides of the tubes, particularly when the cells are in solutions containing formamide. This first wash step is usually where we observe the greatest loss of cells.
15. When performing RNA FISH in solution, it is difficult to aspirate all the hybridization solution without taking some of the pellet as well. We find it is easier to leave some amount of the hybridization solution. Diluting the remaining solution with wash buffer and pelleting will result in a more stable pellet so the solution can be aspirated more easily.
16. If you don't observe a pellet at this step, use a smaller amount of glucose oxidase solution (10–15 μL). Even with no visible pellet, there are often enough cells remaining for successful imaging. You can vary the amount of glucose oxidase solution used to resuspend the cells depending on the size of the pellet and the desired density of cells in the field of view while imaging. It is easier to start with a small volume; if the cells are too dense after mounting and imaging a sample, dilute the remaining cells with more glucose oxidase solution and prepare a new slide.
17. When mounting samples under larger cover slips, increase the amount of cell suspension used. You basically want to use the smallest volume possible so that the liquid spreads out and completely fills the space under the cover slip. Excess will be lost and wicked away into a Kimwipe when the cover slip is pressed down.

18. Squeezing the cells improves imaging of "thick" cells (like mouse embryonic stem cells) by decreasing the depth of field required to fully image the cells. Squeezing also insures that cells are close to the surface of the cover slip. It is not necessary to smash them excessively. Sliding the cover slip from side to side horizontally over the surface of the slide will shear the cells.
19. Avoid sealing with nail polish if possible. It can wick under the cover slip and lead to background in the Cy3/Quasar 570 channel. Rubber cement causes background in the DAPI channel but will not seep under the cover slip. If rubber cement gets on the surface of the cover slip, allow it to dry completely. It is usually possible to take a dry Kimwipe and *gently* remove the rubber cement from the imaging surface. Be careful not to tear the seal around the edges of the cover slip, or the liquid will evaporate. If the rubber cement seal at the edges of the cover slip is torn, it can be reapplied.
20. We do all of our imaging on an inverted microscope which is required to image cells in chambered cover glasses. Adjust the protocol accordingly if imaging on an upright microscope.
21. For very intense signals (XIST, MALAT1, etc.) the exposure time might need to be set to less than 2 s, but you risk missing seeing single-molecule spots. Adjust the contrast to see if single-molecule spots are visible before decreasing the exposure time. Keep in mind that bright nuclear "blobs" might be legitimate signal or they could be caused by off-target binding. You don't want to lower the exposure to get a good picture of off-target binding and miss the real signal.
22. Some cells, particularly stressed cells, have higher levels of background autofluorescence than others. This autofluorescence usually has a broader emission spectrum than the fluorophores and can be seen in multiple channels. For this reason, we like to take a stack of images in a channel (typically corresponding to GFP, where autofluorescence is brighter) that doesn't contain probe so we can compare to channels with RNA FISH signal. If background is interfering with RNA FISH signal, it is sometimes possible to take two sets of stacks starting from the same position and get the background to photobleach more than the probes. Whether this approach works seems to be sample dependent. In this case it is important to carefully check to make sure you aren't losing signal as well. A computational approach to compare spots between RNA FISH channels and the background channel may be preferable/required to subtract punctate background.
23. In some cases a clear threshold between signal and noise cannot be inferred (Fig. 3a). In other cases we found that an apparent clear signal with a full probe might not be validated by the

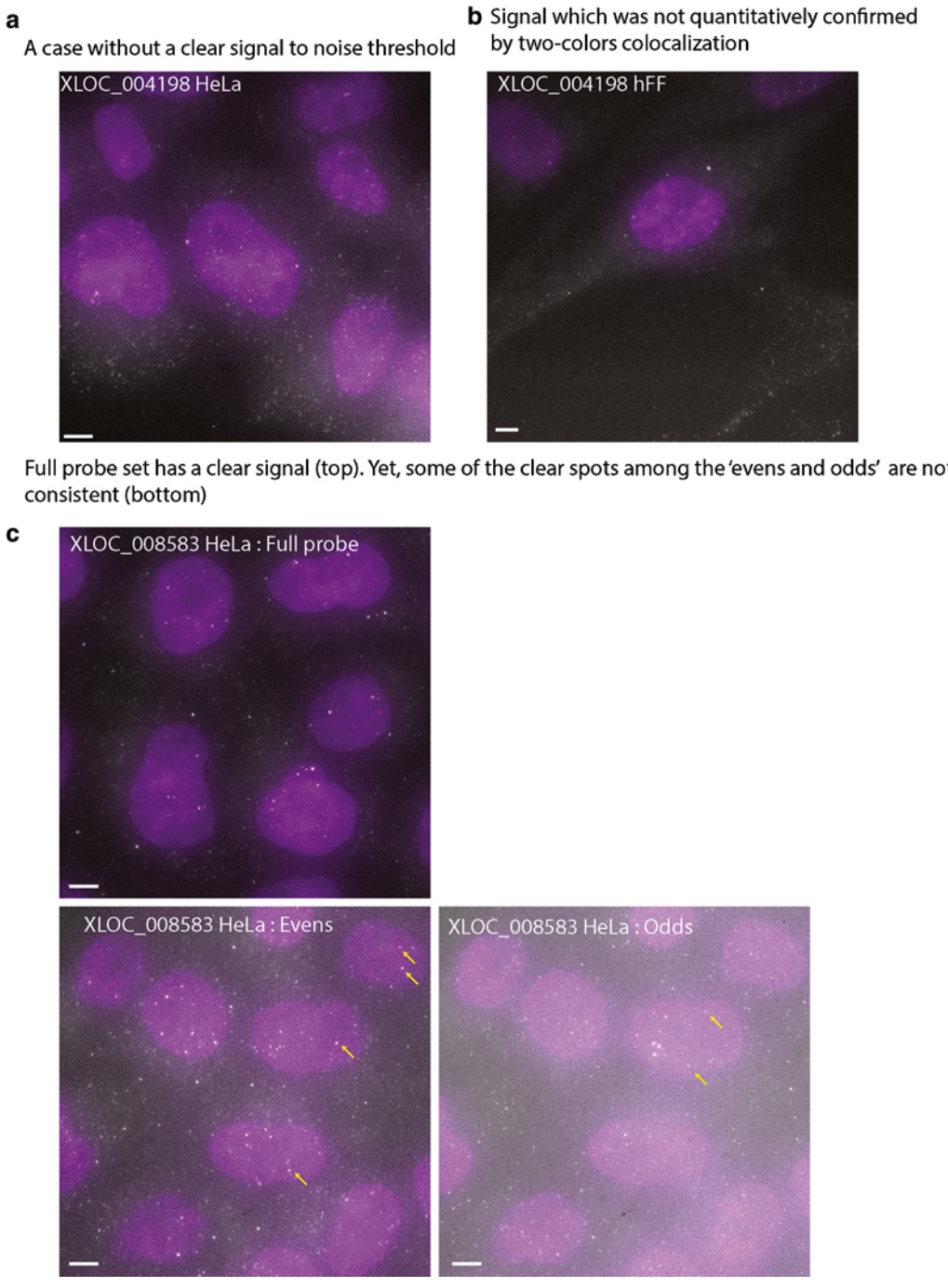

Fig. 3 (**a**) Representative example of a probing of a lncRNA in which a clear signal-to-noise threshold cannot be inferred. (**b**) Example of signal obtained using the full probe set that was not validated used the two-color colocalization assay. (**c**) Example of clear lncRNA signal with a full probe set (*top left*) that was not validated and was quantitatively different than the number of colocalized spots found in the two-color colocalization assay presented in the *bottom* images (evens, *left*; odds, *right*). There are spots with a clear signal that are found with one probe subset and not the other. Representative examples are marked by *yellow arrows*. Scale bar, 5 μm

colocalization approach (Fig. 3b). We also found cases where the whole probe is not quantitative, since some detectable spots only appear on one channel and not the other in the "odds and evens" colocalization assay (Fig. 3c).

24. Frequently, off-target binding of a single oligonucleotide will cause a very high intensity spot in the nucleus. When this is seen in mRNA FISH, you can consider it highly likely spurious; however, many legitimate lncRNA FISH signals look like this (Fig. 4). If you see such a signal in either of the odds or evens channels and lower intensity spots in the other channel that do not colocalize, but look like they could be signal, adjust the contrast and compare the images. Sometimes the auto-contrast of the channel with the high intensity spot will obscure legitimate lower intensity spots that do colocalize.
25. The software is designed to quantify individual mRNA FISH spots and will not work well for highly abundant noncoding transcripts that localize in large "blobs" such as XIST or MALAT1.

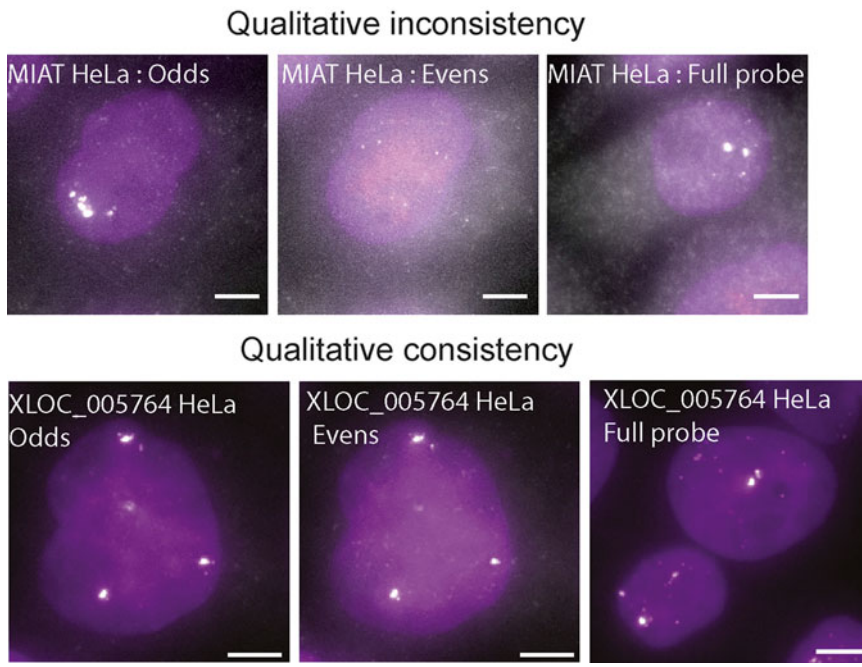

Fig. 4 Bright nuclear foci as invalid and valid signals. *Top*: example of nuclear foci that are seen using the odd-numbered subset (*left*), but not the even-numbered subset (*middle*), and dominate the signal using the full probe (*right*). *Bottom*: example of nuclear foci seen using the full probe set (*right*), which are also validated both in the "even" (*middle*) and "odd" (*left*) subsets

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Chapter 2

Super-Resolution Imaging of Nuclear Bodies by STED Microscopy

Yasushi Okada and Shinichi Nakagawa

Abstract

The sizes of nuclear bodies and other nuclear structures are normally no more than a few hundred nanometers. This size is below the resolution limit of light microscopy and thus requires electron microscopy for direct observation. Recent developments in super-resolution microscopy have extended the resolution of light microscopy to beyond 100 nm. Here, we describe a super-resolution technique, gated STED, for the analysis of the structure of nuclear bodies, with emphasis on the sample preparation and other technical tips that are important to obtain high-quality super-resolution images.

Key words Super-resolution microscopy, Gated STED, Nuclear structures

1 Introduction

Immunofluorescence microscopy enables sensitive and precise localization of specific proteins and other antigens. Fluorescence in situ hybridization (FISH) supplements immunofluorescence for the detection and localization of RNA in cells and tissues, while green fluorescent protein (GFP) and its variants have extended the application of fluorescent microscopy to the live imaging of protein dynamics. Accordingly, the structure and dynamics of nuclear bodies have been examined using these fluorescence-based light microscope techniques. However, the resolution of a light microscope is limited to about 200 nm by diffraction [1], which is comparable or larger in size than most nuclear bodies and other nuclear structures. Therefore, the fluorescent images are blurry, making it impossible to examine details of the structures.

Recently, super-resolution microscopy has successfully extended the resolution to beyond the diffraction limit. Currently three different super-resolution microscope technologies are commercially available. Among them, SLM (single-molecule localization microscopy), is extensively used for the observation of

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structures close to the cover glass [2–5]. SLM is based on precisely determining the positions of fluorescent molecules as the centers of blurry fluorescent spots if the images of the fluorescent molecules are not overlapping. Furthermore, the accuracy of the localization can be better than 10 nm [6] if the signal-to-noise ratio of the image is sufficient [7]. Thus, a super-resolution image can be reconstructed by plotting the position of all the fluorescent molecules when the molecules are sequentially turned on so that each frame of the image has only a small number of nonoverlapping fluorescent spots. The result is that SLM can reach about ten times higher resolution than the diffraction limit, but at the same time can only capture samples near the surface of the cover glass, because it depends on total internal reflection illumination to obtain a high signal-to-noise ratio. Consequently, nuclear bodies and other nuclear structures located deep inside the cell are hard to observe.

A second super-resolution microscope technique, SIM (Structured Illumination Microscopy), is named for its use of stripe patterns for the illumination [8]. Structural details beyond the diffraction limit are recorded as moiré patterns, and twice the resolution of the diffraction limit can be achieved. Although the resolution is limited (about 120 nm), SIM is compatible with all fluorescent dyes and can be applied to deeper structures like the nucleus.

The third super-resolution technique is STED (STimulated Emission Depletion). It is based on confocal laser scanning microscopy (CLSM) and can be applied to thicker samples with optical sectioning capabilities. The resolution of CLSM is limited by the size of the excitation laser spot, which is also limited by diffraction. STED uses a second beam with a donut shape to erase the fluorescence emission by a photochemical process called stimulated emission. Thus, only fluorescent dyes in the hole of the donut remain fluorescent, effectively narrowing the fluorescent spot [9]. Recently, an improved version of STED, gated STED, has been proposed and uses the difference in the fluorescence lifetime between the fluorescent dyes in the donut and those in the hole [10]. Since shortening the lifetime is much easier than erasing the fluorescence, gated STED is compatible with a wider selection of fluorescent dyes and can achieve higher resolution than conventional STED.

As summarized in Table 1, these three super-resolution techniques have their strengths and weaknesses, with STED being most suitable for the observation of fine structures within the nucleus, a location normally distant from the cover glass surface. Therefore, in the following sections, we focus on the preparation of samples for observation with STED.