Published in final edited form as:

Methods Mol Biol. 2021 ; 2372: 123–144. doi:10.1007/978-1-0716-1697-0_12.

Highly Resolved Detection of Long Non-coding RNAs In Situ

Megan Trotter, Clair Harris, Marissa Cloutier, Milan Samanta, Sundeep Kalantry

Department of Human Genetics, University of Michigan Medical School, Ann Arbor, 48105-1536, MI, USA.

Abstract

Long non-coding RNAs (lncRNAs) have been postulated to function in a number of DNA-based processes, most notably transcription. The detection of lncRNAs in situ can offer insights into their function. Fluorescence in situ hybridization (FISH) enables the detection of specific nucleic acid sequences, including lncRNAs, within individual cells. Current RNA FISH techniques can inform both the localization and expression level of RNA transcripts. Together with advances in microscopy, these in situ techniques now allow for visualization and quantification of even lowly expressed or unstable lncRNAs. When combined with detection of associated proteins and chromatin modifications by immunofluorescence, RNA FISH can lend essential insights into lncRNA function. Here, we describe an integrated set of protocols to detect, individually or in combination, specific RNAs, DNAs, proteins, and histone modifications in single cells at high sensitivity using conventional fluorescence microscopy.

Keywords

Long non-coding RNAs; Fluorescence in situ hybridization; RNA FISH; DNA FISH; Immunofluorescence; Chromatin; Histone modifications

1 Introduction

lncRNAs can recruit proteins, including chromatin modifiers, and alter gene expression states [1, 2]. Some lncRNAs are hypothesized to regulate expression of specific loci [3]. Other lncRNAs are postulated to regulate gene expression of broader domains, including chromosome-wide, as in the case of the *Xist* lncRNA and the inactive X-chromosome [1, 4]. Refinements of fluorescence in situ hybridization (FISH) techniques have resulted in the ability to visualize lncRNAs at their sites of synthesis on the chromosome or site of function in the genome at the single cell resolution in cultured cell lines as well as in tissues [5]. More recently, single-molecule and allele-specific RNA FISH procedures have permitted imaging and quantifying RNA molecules at the single allele resolution throughout the cell [6]. Combined with immunofluorescence (IF) detection of chromatin modifying factors and histone modifications, FISH techniques can permit the formulation of specific hypotheses of lncRNA function. For example, colocalization of chromatin modifiers or

histone modifications with lncRNA transcripts can suggest the recruitment of the specific chromatin modifying factors to discrete loci in the genome by these transcripts.

Here, we describe the detection via FISH of lncRNAs with probes that do not distinguish the DNA strand of synthesis, with strand-specific probes, and also with allele-specific probes. We additionally describe methods for IF-based detection of proteins and histone modifications and a DNA FISH procedure to mark specific DNA loci of interest in cultured and embryonic cells. These protocols can be used individually or in combination. We provide examples of cells profiled by IF and RNA FISH, RNA FISH followed by DNA FISH, and allele-specific RNA FISH interrogating the X-linked *Xist* and *Tsix* lncRNAs that are expressed from and function *in cis* on the X-chromosome along with histone H3 lysine 27 trimethylation (H3K27me3), which, like the *Xist* lncRNA, marks the inactive X-chromosome.

2 Materials

2.1 Sample Preparation: Cultured Cells

1. Sterile, gelatinized glass coverslips (*see* Note 1).
2. UltraPure DNase/RNase-free water.
3. 0.2% gelatin (Sigma#G2500): 0.2 g gelatin per 100 ml of UltraPure DNase/RNase-free water. Autoclave to dissolve and sterilize.
4. 6-well tissue culture dish.
5. Cytoskeletal buffer (CSK): 100 mM NaCl, 300 mM sucrose, 3 mM magnesium chloride, 10 mM PIPES (C₈H₁₈N₂O₆S₂, pH 6.8; be sure to avoid using the sodium-salt version of PIPES). For PIPES, make 0.25–0.5 M stock; bring into solution by adding NaOH while measuring pH.
6. CSK buffer with 0.4% Triton X-100. Make stock of 10–20% Triton X-100 solution in UltraPure DNase/RNase-free water before adding to CSK.
7. 4% paraformaldehyde (PFA; Electron Microscopy Science 16% paraformaldehyde, diluted in UltraPure DNase/RNase-free water with a final concentration of 1× phosphate buffered saline [PBS]).
8. 70% ethanol made with filtered ddH₂O.
9. Plate sealer tape.

2.2 Sample Preparation: Mouse Embryos/Embryo Fragments

1. UltraPure DNase/RNase-free water.

¹To prepare coverslips for tissue cultured cells, sterilize coverslips one at a time by dipping in 100% ethanol and running through a flame. Once the coverslip is sterilized in this manner, place it in a well of the 6-well dish. In the tissue culture hood, pipette 0.5–1 ml sterile 0.2% gelatin onto the coverslip and spread around. Keep the gelatin on the surface of the coverslip for ~10 min and avoid letting the gelatin infiltrate between the coverslip and the bottom of the well, or the coverslip may stick to the tissue culture surface. Aspirate excess gelatin and let dry.

2. 0.01% poly-L-lysine (PLL; Sigma #P4707, 0.1% diluted 1:10 in DNase/RNase-free water).
3. PLL-coated glass coverslips (*see* Note 2).
4. Pipette for plating embryos or embryo fragments: A glass Pasteur pipette pulled to a width only slightly larger than the embryos/embryo fragments.
5. 1× PBS with 6 mg/ml of bovine serum albumin (BSA). To make 50 ml, combine 4 ml BSA (Invitrogen, 7.5 g/100 ml #15260037), 5 ml 10× PBS, and 41 ml UltraPure DNase/RNase-free water.
6. Fixation and Permeabilization Solution: 1% PFA with 0.05% tergitol in a final concentration of 1× PBS.
7. 1% PFA (PFA; Electron Microscopy Science 16% paraformaldehyde in aqueous solution, diluted in UltraPure DNase/RNase-free water and with a final concentration of 1× phosphate buffered saline [PBS]).
8. 70% ethanol made with filtered ddH₂O.
9. 6-well dish, or similar container for storage (*see* Note 3).
10. Plate sealer.

2.3 Immunofluorescence (IF)

1. UltraPure DNase/RNase-free water.
2. 1× PBS.
3. 6-well dish, or similar chamber to be used for washing coverslips (*see* Note 3).
4. Blocking Buffer: 1× PBS with 0.5 mg/ml BSA, 50 µg/ml tRNA, 80 units/ml RNase inhibitor (such as RNaseOUT, Invitrogen #10777019), and 0.2% Tween-20 (make and use a 10% Tween-20 stock). Pre-warm to 37 °C.
5. Primary antibody against antigen of choice.
6. Small glass plate (*see* Note 4).
7. Parafilm.
8. Forceps.
9. IF chamber: a small humid chamber for incubating slides, humidity provided by 1× PBS (*see* Note 5).

²-To prepare coverslips for embryos, place glass coverslip in a well of a 6-well dish. Add 2 ml of 0.01% poly-L-lysine, ensure coverslip is submerged and incubate for 15 min while rocking. Wash 3× in DNase/RNase-free water or 1×PBS. Allow coverslips to dry for at least 2 h in a microtube storage box.

³-Use 22 × 22 mm coverslips, which fit well within a single well of a 6-well dish. All the dehydration and washing steps can be performed in the wells of this dish.

⁴-Short glass plates designed for casting protein gels are a good size for the hybridization. For example, BioRad's Mini-PROTEAN Short Plates fit well in the humid chamber described in Note 5.

⁵-For humid chambers, a microscope slide box (i.e., one that holds 100 slides) works well. Place paper towels soaked in 1×PBS/0.2% Tween 20 in the bottom of the box to create a humid chamber for immunofluorescence. For RNA and DNA FISH hybridization procedures, create a humid chamber by placing paper towels soaked in 2× SSC/50% formamide in the bottom of the box.

10. Incubator set to 37 °C.
11. 1× PBS with 0.2% Tween-20.
12. Fluorescently conjugated secondary antibody. AlexaFluor (Invitrogen) secondary antibodies work well with this protocol; AF488, AF555, and AF647 have very similar absorption and emission spectra to the Fluorescein-12, Cy3, and Cy5 dyes used for fluorescence in situ hybridization (FISH), respectively. These antibodies can be used in conjunction with FISH probes for multi-color imaging with the same set of fluorescence microscope filters.
13. 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen, 10 mg reconstituted in ddH₂O to 5 mg/ml) (for IF without subsequent RNA FISH).
14. Mounting medium (for IF without subsequent RNA FISH, *see* Note 6).
15. Microscope slides (for IF without subsequent RNA FISH).
16. 2% PFA (for IF followed by RNA FISH only).

2.4 RNA and DNA FISH: Probe Labeling for Double-Stranded Probes (See Note 7)

1. BioPrime DNA Labeling System (Invitrogen, #18094011) (*see* Note 8).
2. UltraPure DNase/RNase-free water.
3. TE Buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8 (*see* Note 9).
4. DNA template for labeling. Large templates, such as fosmids or BACs, work best for labeling by random priming. BACs are recommended for DNA FISH probes, as the larger template size will maximize the signal despite low copy number of DNA compared to transcribed RNA in the cell. Ensure that the template preparation is extremely pure; contaminating bacterial DNA will lead to high levels of background fluorescent signal.
5. Custom dNTP mixture:
 - a. For use with Cy3 or Cy5 fluorescently labeled dCTP (*see* below): 2 mM dATP, 2 mM dGTP, 2 mM dTTP, 1 mM dCTP.
 - b. For use with Fluorescein-labeled dUTP (Fluorescein-12-dUTP; *see* below): 2 mM dCTP, 2 mM dATP, 2 mM dGTP, 1 mM dTTP.

⁶For mounting medium, use Vectashield (Vector Labs) or similar anti-fade mounting medium and seal coverslips with clear nail polish after mounting on slides.

⁷For “double-stranded” probes (*see* Subheadings 2.4 and 3.4), whereas the labeled probes are single-stranded, the probes are created from a double-stranded fosmid or BAC template using random primers. As such, these probes will hybridize to both strands of DNA and to sense and antisense RNAs transcribed from the same genomic sequence. Double-stranded probes can be used for DNA FISH and for RNA FISH for most genes. With strand-specific probes (*see* Subheadings 2.5 and 3.5), only one strand is labeled, and the probe will hybridize only to the complementary RNA molecule. The strand-specific probes can be used to distinguish sense and antisense transcription from a single locus.

⁸This kit was originally designed for the preparation of biotinylated probes. The protocol outlined above modifies the BioPrime labeling procedure to generate fluorescently labeled probes instead. Do not use the supplied 10× dNTP Mixture, which includes biotinylated nucleotides. Prepare the dNTP mixture and add the fluorescently labeled nucleotides separately as described in Subheading 3.4, **steps 5** and **6**. Alternative random primer solutions have been tested, but do not work as well as the 2.5× Solution provided with this kit.

⁹While these solutions can be prepared from scratch, purchasing them is preferred to ensure their DNase/RNase-free purity.

6. 1 mM fluorescently labeled nucleotide: Fluorescein-12-dUTP (Roche) labeled probes excite maximally at 495 nm and emit maximally at 521 nm. Cy3-labeled dCTP (GE Healthcare) labeled probes excite maximally at 550 nm and emit maximally at 570 nm, while Cy5-labeled dCTP (GE Healthcare) labeled probes absorb maximally in the far-red end of the spectrum, at 649 nm, and emit maximally at 670 nm. The spectra for these dyes do not overlap significantly and labeled probes can be combined with other FISH probes and used with DAPI, which absorbs maximally at 358 nm and emits maximally at 461 nm when bound to double-stranded DNA, or fluorescently conjugated antibodies for multi-color imaging.
7. G-50 ProbeQuant Micro Columns (GE Healthcare).
8. Tabletop centrifuge.
9. 20 mg/ml yeast tRNA: reconstitute lyophilized yeast tRNA (Invitrogen, #15401) in UltraPure DNase/RNase-free water. Aliquot and store at -20°C .
10. 3 M sodium acetate, pH 5.2 (*see* Note 9).
11. Molecular biology grade 100% ethanol.
12. Heat block set to 37°C .

2.5 RNA FISH: Probe Labeling for Strand-Specific Probes (See Note 7)

1. MAXIscript T3/T7 Kit (Ambion, AM1326).
2. Linear DNA template, PCR amplified with T3 or T7 promoter sequence upstream of the sequence to be labeled. To detect RNA transcribed from the desired strand, the probes should be complementary to the target transcript; the T3 or T7 RNA polymerase promoter sequences must therefore be incorporated at the appropriate ends of the template DNA. See MAXIscript kit instructions for details on template preparation.
3. Custom NTP mixture (*see* Note 10):
 - a. For use with Cy3 or Cy5 fluorescently labeled CTP (*see* below): 2 mM ATP, 2 mM GTP, 2 mM TTP, 1 mM CTP.
 - b. For use with Fluorescein-labeled UTP (Fluorescein-12-UTP, *see* below): 2 mM CTP, 2 mM ATP, 2 mM GTP, 1 mM TTP.
4. 100 nmol fluorescently labeled nucleotide: Fluorescein-12-UTP (Roche), Cy3-CTP (GE Healthcare), or Cy5-CTP (GE Healthcare) (*see* notes on emission spectra in Subheading 2.4).
5. 0.5 M EDTA (*see* Note 9).

¹⁰A set of 10 mM NTPs and the T3 and T7 enzymes are included in the MAXIscript kit. Both the NTPs and enzymes can be replaced with other commercially available substitutes with no apparent loss of enzymatic activity.

6. 20 mg/ml yeast tRNA: reconstitute lyophilized yeast tRNA in UltraPure DNase/RNase-free water. Aliquot and store at -20°C .
7. 5 M ammonium acetate (*see* Note 9).
8. 100% molecular biology grade ethanol.
9. UltraPure DNase/RNase-free water.
10. Heat block set to 37°C .
11. Mini Quick Spin RNA Columns (Roche, #11814427001).
12. Tabletop centrifuge.

2.6 RNA and DNA FISH: Probe Precipitation

1. Fluorescently labeled probes in ethanol (*see* Subheadings 3.4 and 3.5). A combination of double-stranded and strand-specific probes may be used.
2. 20 mg/ml yeast tRNA: reconstitute lyophilized yeast tRNA in UltraPure DNase/RNase-free water. Aliquot and store at -20°C .
3. 1 mg/ml COT-1 DNA (Invitrogen) (Optional, *see* Note 11).
4. 10 mg/ml salmon sperm DNA.
5. 5 M ammonium acetate (*see* Note 9).
6. UltraPure DNase/RNase-free water.
7. 100% molecular biology grade ethanol.
8. Tabletop centrifuge.
9. 70% ethanol made with filtered ddH₂O.
10. Deionized formamide (Amresco).
11. 50% dextran sulfate (Millipore).
12. 20× saline sodium citrate (SSC) (Invitrogen).
13. 2× hybridization Solution: 200 μl UltraPure DNase/RNase-free water, 100 μl 20× SSC, 200 μl 50% dextran sulfate (*see* Note 12).
14. Vacuum centrifuge.
15. Heat block set to 90°C .

¹¹-COT-1 DNA can be added to double-stranded probes to help reduce background by hybridizing to repetitive sequences, if necessary. COT-1 DNA should not be used in strand-specific probes, as these probes are specific to the target sequence. While routine use of mouse COT-1 in the precipitation of mouse probes does not appear to be necessary, COT-1 use may reduce hybridization of FISH probes to repetitive sequences. If COT-1 is omitted, pre-annealing is not required.

¹²-50% Dextran sulfate is very viscous. Make the hybridization solutions in a tube with graduations. Pipet all the reagents into the tube other than the dextran sulfate. Then, instead of pipetting the dextran sulfate, use a pipette tip to “scoop” it into the tube up to the appropriate measurement marking on the side of microtube. Vortex thoroughly to mix.

2.7 Allele-Specific RNA FISH: Guide Probe Precipitation

1. Fluorescein-12-UTP labeled strand-specific probe in ethanol (*see* Subheading 3.5).
2. UltraPure DNase/RNase-free water.
3. 5 M ammonium acetate.
4. Molecular biology grade 100% ethanol.
5. Tabletop centrifuge.
6. 70% ethanol made with filtered ddH₂O.
7. Vacuum centrifuge.
8. Deionized formamide (Amresco).
9. 50% dextran sulfate (Millipore).
10. 20× saline sodium citrate (SSC) (Invitrogen).
11. Heat block set to 90 °C.
12. Allele-specific hybridization solution: 300 µl UltraPure DNase/RNase-free water, 50 µl 20× SSC, 50 µl deionized formamide, 100 µl 50% dextran sulfate (*see* Note 12).

2.8 RNA FISH: Sample Hybridization

1. 100% molecular biology grade ethanol. Use filtered ddH₂O to make stocks of 70%, 85%, and 95% ethanol.
2. Fluorescently labeled probes (*see* Subheadings 3.4, 3.5, and 3.6).
3. 6-well dish, or similar chamber to be used for dehydration and for washing coverslips (*see* Note 3).
4. Small glass plate (*see* Note 4).
5. Parafilm.
6. Forceps.
7. Hybridization chamber: a small humid chamber for incubating slides, humidity provided by 2× SSC/50% deionized formamide (*see* Note 5).
8. Incubator set to 37 °C, for overnight incubation.
9. 2× SSC/50% deionized formamide: 5 ml 20× SSC, 25 ml deionized formamide, 20 ml filtered ddH₂O.
10. 2× SSC: 5 ml 20× SSC, 45 ml filtered ddH₂O.
11. 1× SSC: 2.5 ml 20× SSC, 47.5 ml filtered ddH₂O.
12. DAPI (Invitrogen, 10 mg reconstituted in ddH₂O to 5 mg/ml).
13. Incubator set to 39 °C, for washes.

14. Mounting medium (*see* Note 6).
15. Microscope slides.

2.9 DNA FISH: Sample Hybridization

1. 1× PBS.
2. 1% PFA with 0.5% Tergitol and 0.5% Triton X-100. (For DNA FISH following RNA FISH)
3. 100% molecular biology grade ethanol. Use filtered ddH₂O to make stocks of 70%, 85%, and 95% ethanol.
4. 6-well dish, or similar chamber to be used for dehydration and for washing coverslips (*see* Note 3).
5. RNase A solution: 1.25 µg/µl RNase A, diluted in 2× SSC.
6. 2× SSC/70% deionized formamide: 10 µl 20× SSC, 70 µl deionized formamide, 20 µl filtered ddH₂O.
7. Heat block set to 95 °C.
8. Fluorescently labeled double-stranded probe (*see* Subheadings 3.4 and 3.6).
9. Small glass plate (*see* Note 4).
10. Parafilm.
11. Forceps.
12. Hybridization chamber: a small humid chamber for incubating slides, humidity provided by 2× SSC/50% deionized formamide (*see* Note 5).
13. Incubator set to 37 °C, for overnight incubation.
14. 2XSSC/50% deionized formamide: 5 ml 20× SSC, 25 ml deionized formamide, 20 ml filtered ddH₂O.
15. 2× SSC: 5 ml 20× SSC, 45 ml filtered ddH₂O.
16. 1× SSC: 2.5 ml 20× SSC, 47.5 ml filtered ddH₂O.
17. DAPI (Invitrogen, 10 mg reconstituted in ddH₂O to 5 mg/ml).
18. Incubator set to 37 °C, for washes.
19. Mounting medium (*see* Note 6).
20. Microscope slides.

2.10 Allele-Specific RNA FISH Protocol: Sample Hybridization (See Note 13)

1. 6-well dish, or similar chamber to be used for dehydration and for washing coverslips (*see* Note 3).
2. Forceps.

3. 100% molecular biology grade ethanol. Use filtered ddH₂O to make stocks of 70%, 85%, and 95% ethanol.
4. Single-stranded Fluorescein-12-UTP labeled probe prepared for allele-specific FISH (*see* Subheading 3.7), detecting the same transcript as the Quasar dye labeled probe mixes in **steps 5** and **6**.
5. 0.4 μ M probe mix for the reference allele: Equal mixture of 5 short oligonucleotide probes overlapping a SNP specific to one allele of the hybrid Samples being analyzed. Each oligonucleotide is labeled with Quasar 570, which excites maximally at 548 nm and emits maximally at 566 nm. Quasar 570 labeled probes will appear in the same channel as Cy3 and cannot be used in conjunction with a Cy3-labeled probe. Use at a final dilution of 8 nM for hybridization (Biosearch Technologies, *see* Note 14).
6. 0.4 μ M probe mix for alternate allele: Equal mixture of 5 short oligonucleotide probes overlapping a SNP specific to one allele of the hybrid Samples being analyzed. Each oligonucleotide is labeled with Quasar 670, which excites maximally at 647 nm and emits maximally at 670 nm. Quasar 670 labeled probes will appear in the same channel as Cy5 and cannot be used in conjunction with a Cy5-labeled probe. Use at a final dilution of 8 nM for hybridization (Biosearch Technologies, *see* Note 14).
7. 1.2 μ M mask probe mix. Use at a final dilution of 24 nM for hybridization (Biosearch Technologies, *see* Note 15).
8. Incubator set to 37 °C.
9. Hybridization chamber (*see* Note 5).
10. Parafilm.
11. Small glass plate (*see* Note 4).
12. 2 $\times$ SSC/10% deionized formamide: 5 ml 20 $\times$ SSC, 5 ml deionized formamide, 40 ml filtered ddH₂O.
13. 2 $\times$ SSC (Invitrogen).

¹³. Allele-specific FISH relies on hybrid Samples with known single nucleotide polymorphisms (SNPs) between the two parental strains. Probes designed to bind variant nucleotides at the SNP sites will only detect a signal from RNA transcripts originating from one or the other parental allele and can be utilized to examine expression of imprinted genes or other genes that are monoallelically expressed. This protocol is specific for Xist RNA but can be modified for use with any long non-coding RNA (lncRNA) with allele-specific expression.

¹⁴. See refs. 6, 7 for methodology for the design of allele-specific probes. Briefly, in the example for allele-specific detection of Xist RNA, a panel of short oligonucleotide probes was designed to uniquely detect either the *M. musculus* or the *M. molossinus* alleles of Xist RNA [6]. Each oligonucleotide probe overlaps a SNP site that differs between the two strains, with the SNP located at the fifth base pair position from the 5' end. The five such probes used were mixed together in equal proportions to be used in the FISH hybridization. Probes overlapping both the reference and the alternate nucleotides at each of the five SNP sites are used to distinguish allelic expression. The 3' end of each oligonucleotide probe is fluorescently tagged using Quasar dyes (Biosearch technologies).

¹⁵. In addition to labeled SNP-overlapping oligonucleotides, a panel of 5 "mask" oligonucleotides were also synthesized. These "mask" probes are complementary to the 3' end of the labeled allele-specific probes and will hybridize to the allele-specific oligonucleotides, leaving available only 9–10 base pairs of sequence adjacent to the polymorphic site to initially hybridize to the target RNA. Since this region of complementarity is short, the presence of a SNP is sufficient to destabilize the hybridization of the probe to the alternate allele. It is important that the mask probe is used at a higher concentration than the allele-specific probes to ensure that the mask probe is saturating.

14. DAPI (Invitrogen, 10 mg reconstituted in ddH₂O to 5 mg/ml).
15. Mounting medium (*see* Note 6).
16. Clear nail polish.
17. Microscope slides.

3 Methods

3.1 Sample Preparation: Cultured Cells (See Note 16)

1. Grow cells to desired confluency on sterile, gelatinized glass coverslips (*see* Note 1) in the bottom of a 6-well tissue culture dish.
2. Prepare reagents for fixation and permeabilization: make and sterile filter CSK and CSK with 0.4% Triton X-100 buffers and chill to 4 °C. Prepare 4% PFA. All subsequent steps should be performed in the tissue culture hood. The CSK and CSK + 0.4% Triton X-100 buffers should be kept chilled on ice during the procedure.
3. Aspirate media from all six wells of a 6-well dish.
4. Pipette 2 ml of ice-cold CSK buffer in each well of the 6-well dish.
5. After 30 s, aspirate the CSK buffer: by the time CSK buffer has been pipetted into each of the 6 wells, the first well has incubated in the CSK buffer for ~30 s, so simply pipette CSK buffer into the 6 wells and then aspirate right afterward from the first well onward.
6. Pipette 2 ml of ice-cold CSK + 0.4% Triton X-100 buffer in each well of the 6-well dish.
7. After 30 s, aspirate the CSK + 0.4% Triton X-100. See details in **step 5**.
8. Pipette 2 ml of ice-cold CSK buffer in each well of the 6-well dish.
9. After 30 s, aspirate the CSK buffer. See details in **step 5**.
10. Pipette 2 ml of 4% PFA in each well of the 6-well dish. Incubate the cells in PFA for 10 min.
11. Aspirate the PFA and pipette in 5 ml of cold 70% ethanol in each well. Repeat the ethanol wash a total of 3 times, to remove all traces of PFA.
12. Seal dish with plate sealer tape and store in 5 ml 70% ethanol at –20 °C (*see* Note 17).

¹⁶Washing cells with CSK buffers will extract soluble RNAs and proteins from the cytoplasm. This treatment will reduce background and yield cleaner results in RNA FISH stains when detecting nascent or stabilized RNAs. For staining of cytoplasmic or soluble RNAs and proteins, or for quantification of single-molecule RNA FISH signals, CSK buffer should not be used. Instead, Samples can be permeabilized with detergents such as 0.5% Triton X-100 for 5 min on ice.

¹⁷Fixed cells and embryos on coverslips in 70% ethanol can be stored in –20 °C for at least 1 year. Seal with plate sealer tape to minimize evaporation of ethanol and replace the ethanol in the wells occasionally (once every 2 months or so), as it will evaporate over time despite the plate sealer.

3.2 Sample Preparation: Mouse Embryos

1. Plate embryos or dissected embryo fragments on sterile, PLL-coated glass coverslips (22 × 22 cm square coverslips, *see* Note 2) in 1× PBS 6 mg/ml BSA using finely pulled Pasteur pipette.
2. Aspirate excess 1× PBS 6 mg/ml BSA and let dry for about 20–30 min.
3. Fix and permeabilize with 50 µl of 1% PFA in 1× PBS with 0.05% Tergitol for 5 min (to get rid of excess solution on coverslip after the 5 min permeabilization/fixation, simply tap solution off onto a paper towel).
4. Fix again with 50 µl of 1% PFA (no Tergitol) in 1× PBS for 5 min. Drain the excess solution onto a paper towel.
5. Place coverslips with plated embryos in a 6-well dish. Rinse with 3 changes of 70% ethanol.
6. Seal dish with plate sealer tape and store in 5 ml 70% ethanol at –20 °C until use (*see* Note 17).

3.3 Immunofluorescence (IF)

1. Begin with fixed, permeabilized cells or embryo Samples, plated on gelatinized or PLL-coated glass coverslips and stored in 70% ethanol (*see* Subheadings 3.1 and 3.2).
2. Make blocking buffer and warm to 37 °C.
3. Place sample coverslip in a 6-well dish that contains 2 ml of 1× PBS in each well (*see* Note 3).
4. Wash briefly with 3 changes of 1× PBS to remove ethanol.
5. Wash with 1× PBS 3 times, 3 min each on a rocker.
6. Wrap a glass plate tightly with parafilm for incubating coverslips for subsequent steps.
7. Block slides for 30 min at 37 °C in 50 µl pre-warmed blocking buffer in a humid chamber: place a 50 µl drop of blocking buffer on the parafilm-wrapped glass plate and invert the coverslip, sample side down, into the blocking buffer. Place the parafilm-wrapped plate in the humid chamber and incubate for 30 min at 37 °C. All incubations in blocking buffer, primary antibody, or secondary antibody should be set up in this manner.
8. Carefully lift coverslip from blocking buffer with forceps and place sample side down into a 50 µl droplet of primary antibody diluted in pre-warmed blocking buffer (dilution based on primary antibody of choice) on a parafilm-wrapped plate in a humid chamber at 37 °C for 1 h.
9. Remove coverslip from primary antibody solution and place, sample side up, in a 6-well dish. Wash 3 times with 1× PBS/0.2% Tween-20 for 3 min on a rocker.

10. Incubate in 50 μ l pre-warmed blocking buffer on a parafilm-wrapped plate in a humid chamber for 5 min at 37 °C.
11. Incubate with 50 μ l secondary antibody diluted in pre-warmed blocking buffer in humid chamber at 37 °C for 30 min. Antibody dilution depends on secondary antibody used. AlexaFluor conjugated secondary antibodies should be used at a 1:300 dilution.
12. Remove coverslip from secondary antibody and wash 3 times with 1 $\times$ PBS/0.2% Tween-20 for 3 min each on a rocker.
 - a. If processing Samples only for IF, the first wash of **step 12** should contain a 1:100,000 to 1:200,000 dilution of DAPI (*see* Note 18). Then, rinse once briefly with PBS/0.2% Tween-20 and wash 2 more times for 5–7 min each while rocking to remove excess DAPI. Remove coverslip from dish and tap off excess liquid, then mount on a slide, sample side down, in mounting medium. Image Samples or store at –20 °C for later imaging.
 - b. If Samples will be stained by RNA FISH probes following IF, pipette a 100 μ l drop of 2% PFA on a glass plate wrapped in parafilm. After washes, place the coverslip sample side down in PFA and place in the humid chamber. Incubate the sample 10 min at room temperature, then proceed to RNA FISH hybridization (Subheading 3.8).

3.4 RNA FISH: Probe Labeling for Double-Stranded Probes

1. Dilute 50–100 ng DNA template in TE buffer in a final volume of 19 μ l.
2. On ice, add 20 μ l 2.5 $\times$ Random Primers Solution (supplied with BioPrime labeling kit).
3. Denature DNA by heating for 5 min in a 90 °C heat block.
4. Immediately cool on ice for 5 min.
5. Add 5 μ l of custom dNTP mixture.
6. Add 5 μ l of 1 mM fluorescently labeled nucleotide.
7. Add 2 μ l (80 units) Klenow fragment (Supplied with the BioPrime Labeling Kit, *see* Note 19).
8. Incubate at 37 °C overnight. Cover the tube with aluminum foil to protect the probe from light during this step.
9. Add 5 μ l of Stop Buffer.

¹⁸. Dilute DAPI 1:500 in UltraPure DNase/RNase-free water, store in the dark at –20 °C. Add 5 μ l of this dilution into each well holding 2ml 2 $\times$ SSC while washing Samples.

¹⁹. The Klenow Fragment included with the BioPrime kit can be replaced with other commercially available Klenow enzymes with no apparent loss of enzymatic activity.

10. Prepare G-50 column: Vortex briefly to mix the Sephadex beads. Twist off the bottom and place the column in one of the provided collection tubes. Spin at 750 g for 1 min.
11. Move column to a new microcentrifuge tube and pipette the probe into the center of the column (*see Note 20*). Spin at 750 g for 1 min. Measure eluted volume by pipette.
12. Add 10 μ l of 20 mg/ml yeast tRNA.
13. Add 3 M sodium acetate to a final concentration of 0.3 M.
14. Add 2.5 volumes of 100% ethanol.
15. Spin at 4 °C for 20 min at top speed, $\sim 21,100 \times g$.
16. Carefully aspirate the supernatant using the vacuum apparatus (*see Note 21*).
17. Resuspend the pellet in 360 μ l of UltraPure DNase/RNase-free ddH₂O. Make sure the pellet is fully dissolved before moving to the next step.
18. Add 40 μ l 3 M sodium acetate.
19. Add 1 ml 100% ethanol.
20. Store labeled probe at -20 °C in the dark (*see Note 22*).

3.5 RNA FISH: Probe Labeling for Strand-Specific Probes

1. Start with 1 μ g template DNA.
2. Add DNase/RNase-free UltraPure ddH₂O up to 9 μ l, so that the total volume at the end of **step 6** will be 20 μ l.
3. Add 2 μ l 10 $\times$ Transcription Buffer (supplied with MAXIscript kit).
4. Add 5 μ l NTP mixture (supplied with MAXIscript kit, *see Note 10*).
5. Add 2 μ l of fluorescently labeled NTP.
6. Add 2 μ l T3 (60 units) or T7 (30 units) enzyme mix (supplied with MAXIscript kit, *see Note 10*).
7. Mix thoroughly.
8. Incubate for 2 h at 37 °C.
9. Add 1 μ l of TURBO DNase (supplied with MAXIscript kit) and mix well.
10. Add 1 μ l of 0.5 mM EDTA.

²⁰Be sure to pipet the probe into the center of the beads in the column during column purification. The probe must travel through the beads, not down the side.

²¹The pellet is usually attached to the bottom of the tube but be careful during aspiration of the supernatant. A 10 μ l pipette tip can be attached to the end of the aspiration tube to slow suction.

²²Ethanol stocks of probes can last up to 1 year when stored, sealed, and protected from light.

11. Prepare Quick Spin RNA Column: Vortex briefly to mix the Sephadex beads. Remove cap and twist off the bottom tip, then place the column in a microcentrifuge tube. Spin at $1000 \times g$ for 1 min.
12. Move column to a new microcentrifuge tube and pipette the probe into the center of the column (*see* Note 20). Spin at $1000 \times g$ for 1 min. Measure the new volume by pipette.
13. Add 10 μ l of 20 mg/ml yeast tRNA.
14. Add 5 M ammonium acetate to a final concentration of 0.5 M.
15. Add 2.5 volumes of 100% ethanol.
16. Spin for 20 min at 4 °C at top speed, $21,100 \times g$.
17. Carefully aspirate the supernatant using the vacuum apparatus (*see* Note 21).
18. Resuspend the pellet in 360 μ l DNase/RNase-free UltraPure ddH₂O. Make sure the pellet is fully dissolved before moving to the next step.
19. Add 40 μ l of 5 M ammonium acetate.
20. Add 1 ml of 100% ethanol.
21. Store labeled probe at -20 °C in the dark (*see* Note 22).

3.6 RNA FISH: Probe Precipitation

All spin steps should be completed at top speed, $\sim 21,100 \times g$.

1. Aliquot 100 μ l of fluorescently labeled probe in ethanol. A combination of double-stranded and strand-specific probes may be precipitated together (*see* Note 23). Vortex all probe stocks before aliquoting.
2. Add 15 μ l of 20 mg/ml tRNA (300 μ g) (*see* Note 24). Mix thoroughly by pipetting.
3. Add 15 μ l of 1 mg/ml mouse COT-1 DNA (15 μ g). Mix thoroughly by pipetting. (Optional, *see* Notes 11 and 24. If not using COT-1 DNA, add 15 μ l of UltraPure DNase/RNase-free water).
4. Add 15 μ l of 10 mg/ml sheared boiled salmon sperm DNA (150 μ g) (*see* Note 24). Mix thoroughly by pipetting.
5. Add 20 μ l 5 M ammonium acetate (*see* Note 25). Vortex briefly to mix.
6. Add 45 μ l of DNase/RNase-free UltraPure water (*see* Note 25). Vortex briefly to mix.

²³. At this stage, multiple probes that were labeled with different fluorophores can be combined. To start, precipitate 100 μ l ethanol stock of each probe together. If any of the probes are too bright or too faint, the initial starting volume of the ethanol stock of the probes can be adjusted.

²⁴. The volumes of tRNA, Cot-1 DNA, and Salmon sperm DNA (Subheading 3.6, **steps 2–4**) are determined by the final volume of probe, these will stay constant whether precipitating one or multiple probes together.

²⁵. The volumes of ammonium acetate, H₂O, and 100% ethanol (Subheading 3.6, **steps 5–7**) are determined by the starting volume of ethanol. These will need to be scaled up proportionately if multiple probes are precipitated together.

7. Add 250 μ l of 100% ethanol (*see* Note 25). Vortex briefly to mix.
8. Spin for 20–30 min at 4 °C to precipitate probe.
9. Carefully aspirate the supernatant using a vacuum apparatus (*see* Note 21).
10. Add 500 μ l of 70% ethanol. Vortex thoroughly. Spin at room temperature for 4 min.
11. Carefully aspirate the supernatant using a vacuum apparatus (*see* Note 21).
12. Add 500 μ l of 100% ethanol. Vortex thoroughly. Spin at room temperature for 4 min.
13. Carefully aspirate the supernatant using a vacuum apparatus (*see* Note 21).
14. Dry in a speed vacuum without heat for 5 min, or alternatively, air-dry. No ethanol should remain in the tube for the next step.
15. Add 50 μ l of 100% deionized formamide to the tube. Make sure that the pellet is submerged in the formamide.
16. Denature in a heat block at 80–90 °C for 10 min. At ~5 min, pipette the formamide up and down to make sure that the pellet goes into solution. Place back into the heat block.
17. Immediately cool on ice for 5 min.
18. Add 50 μ l 2 $\times$ hybridization solution. Mix thoroughly by pipetting repeatedly (*see* Note 26).
19. Pre-anneal at 37 °C for 1–1.5 h. (Optional, *see* Note 11. Pre-annealing is not required for strand-specific probes or for double-stranded probes precipitated without COT-1 DNA).
20. Store probe at –20 °C in the dark.

3.7 Allele-Specific RNA FISH: Guide Probe Precipitation

1. Aliquot 100 μ l of the fluorescently labeled probe into a clear 1.5 ml tube.
2. Add 260 μ l DNase/RNase-free water. Vortex briefly to mix.
3. Add 40 μ l of 5 M ammonium acetate. Vortex briefly to mix.
4. Add 1 ml of 100% ethanol. Vortex briefly to mix.
5. Spin to precipitate the probe mix at top speed for 20–30 min at 4 °C (*see* Note 27).
6. Aspirate the supernatant using the vacuum apparatus (*see* Note 21).
7. Add 500 μ l of 70% ethanol and spin at top speed for 4 min at room temperature.

²⁶Composition of 2 $\times$ hybridization buffer: 4 $\times$ SSC and 20% dextran sulfate. Final composition of the RNA FISH hybridization buffer after mixing the 2 $\times$ hybridization buffer with an equal volume of the probe in 100% deionized formamide: 50% deionized formamide, 2 $\times$ SSC, and 10% dextran sulfate.

²⁷Be sure to place the hinge of the tube facing out to ensure the location of the pellet once spinning is complete.

8. Aspirate the supernatant using the vacuum apparatus (*see* Note 21).
9. Add 500 μ l of 100% ethanol and spin at top speed for 4 min at room temperature.
10. Aspirate the supernatant using the vacuum apparatus (*see* Note 21).
11. Dry in a speed vacuum without heat for 5 min, or alternatively, air-dry. No ethanol should remain in the tube for the next step.
12. Resuspend pellet in 10 μ l of 100% deionized formamide.
13. Denature at 90 °C for 8 min in heat block. At ~4 min, pipette the formamide up and down to make sure that the pellet goes into solution. Place back into the heat block.
14. Immediately cool on ice for 5 min.
15. Add 90 μ l of allele-specific hybridization solution. Pipette to mix (*see* Note 28).
16. Store precipitated probe at –20 °C.

3.8 RNA FISH: Sample Hybridization

1. Start with permeabilized and fixed Samples plated on coverslips and stored in 70% ethanol (*see* Subheadings 3.1 or 3.2), or Samples previously processed for IF (*see* Subheading 3.3).
2. Dehydrate the coverslips by moving them through a room-temperature ethanol series (85%, 95%, and 100% ethanol) for 2 min each.
3. Remove the coverslips from the well and air-dry at room temperature for 15 min (*see* Note 29).
4. Set up the FISH hybridization. Use 8–10 μ l of probe per 22 $\times$ 22mm coverslip of cells and 12 μ l of probe per coverslip of embryos. Pipette the probe onto a parafilm-wrapped glass plate (*see* Note 4), invert the coverslip onto the droplet of probe, and tap lightly with forceps to help the probe spread across the coverslip.
5. Hybridize overnight at 37 °C in a humid chamber (humidity provided by 2 $\times$ SSC/50% formamide) (*see* Note 5).
6. Make all wash solutions (2 $\times$ SSC/50% formamide, 2 $\times$ SSC, and 1 $\times$ SSC), and warm the solutions to 39 °C.
7. Carefully peel the coverslip off of the parafilm, re-invert and place in a well containing pre-warmed 2 $\times$ SSC/50% formamide. Be sure the coverslip goes into the well with the sample side up.

²⁸Composition of the allele-specific RNA FISH hybridization buffer: 10% deionized formamide, 2 $\times$ SSC, and 10% dextran sulfate. Final composition of the allele-specific RNA FISH hybridization buffer after mixing 90 μ l of the buffer above with 10 μ l probe in 100% deionized formamide: 19% deionized formamide, 1.8 $\times$ SSC, 9% dextran sulfate.

²⁹Do not leave the coverslip to dry in the well after the 100% ethanol is aspirated. The glass coverslip may stick to the bottom of the well. Find a safe place to set the coverslip to dry. Laying them across the inside of an empty pipette tip box or microtube storage box works well, and closing the lid of the box can prevent dust from settling on the coverslip and preserve fluorescent signal.

8. Wash with pre-warmed 2× SSC/50% formamide at 39 °C, 3 times for 7 min each.
9. Wash with pre-warmed 2× SSC at 39 °C, 3 times for 7 min each. Add DAPI into the last 2× SSC wash at a 1:100,000–1:200,000 dilution (*see* Note 18).
10. Rinse once quickly with pre-warmed 1× SSC.
11. Wash with pre-warmed 1× SSC at 39 °C, 2 times for 7 min each.
12. Use mounting medium to mount the coverslip onto a labeled microscope slide. Invert the slide onto a paper towel and press gently but firmly to remove excess mounting medium from under the coverslip.
13. Seal the coverslip and let dry thoroughly before viewing under a microscope.
14. Slides can be stored in the dark at –20 °C to preserve the fluorescent signal.

3.9 DNA FISH: Sample Hybridization

1. Begin with permeabilized and fixed Samples, plated on coverslips and stored in 70% ethanol (*see* Subheadings 3.1 and 3.2) or with Samples previously stained for RNA FISH (*see* Subheading 3.8). For Samples previously stained by RNA FISH, RNA FISH signals will be degraded during DNA FISH, so Samples should be imaged prior to beginning this protocol. Take note of the visual fields imaged, so that RNA FISH images can be aligned with DNA stains later.
2. If using Samples previously stained for RNA FISH, use a razor blade to cut away the nail polish used to seal the coverslip to the slide. Then submerge the entire slide with the coverslip still attached in a solution of 2× SSC. While the sample is still submerged, gently peel off the coverslip by letting the solution of 2× SSC infiltrate between the coverslip and the slide. For Samples not previously processed for RNA FISH, proceed to **step 5**.
3. Wash the coverslip with 1× PBS three times quickly and then incubate in 1× PBS for 5 min at room temperature.
4. Re-fix the cell/embryos with 1% PFA containing 0.5% tergitol and 0.5% Triton X-100. Fix for 10 min at room temperature.
5. Dehydrate the coverslips by moving them through a room-temperature ethanol series (70%, 85%, and 100% ethanol) for 2 min each.
6. Remove the coverslips from the well and air-dry at room temperature for 15 min (*see* Note 29).
7. RNase treatment: Treat with 1.25 µg/µl RNase A in 2× SSC. Invert slides onto a 100 µl drop of RNase A solution on parafilm stretched over a glass slide. Incubate at 37 °C for 30 min. RNase treatment allows the probe to gain access to the DNA.
8. Dehydrate the coverslips by moving them through a room-temperature ethanol series (85%, 95%, and 100% ethanol) for 2 min each.

9. Remove the coverslips from the well and air-dry at room temperature for 15 min (*see* Note 21).
10. Denature the Samples in a pre-warmed solution of 2× SSC/70% formamide on a glass slide or glass plate stationed on top of a heat block set at 95 °C for 11 min. Denaturation time and temperature are sample dependent and need to be optimized (*see* Note 30).
11. Immediately dehydrate through a −20 °C ethanol series (70%, 85%, 95%, and 100% ethanol) for 2 min each.
12. Remove the coverslips from the well and air-dry at room temperature for 15 min (*see* Note 29).
13. Set up the FISH hybridization. Use 8–10 µl of probe per 22 × 22 mm coverslip of cells and 12 µl of probe per coverslip of embryos. Pipette the probe onto parafilm-wrapped glass plate (*see* Note 4), invert the coverslip onto the droplet of probe and tap lightly with forceps to help the probe spread across the coverslip. Be sure to avoid air bubbles that may result in uneven distribution of the probe.
14. Hybridize overnight at 37 °C in a humid chamber (humidity provided by 2× SSC/50% formamide, *see* Note 5).
15. Make all wash solutions (2× SSC/50% formamide, 2× SSC), and warm the solutions to 39 °C.
16. Carefully peel the coverslip off of the parafilm, re-invert and place in a well containing pre-warmed 2× SSC/50% formamide. Be sure the coverslip goes into the well with the sample side up.
17. Wash with 2× SSC/50% formamide at 39 °C twice, 7 min each.
18. Wash with pre-warmed 2× SSC plus a 1:100,000–1:200,000 dilution of DAPI for 7 min at 39 °C (*see* Note 18).
19. Wash with pre-warmed 2× SSC without DAPI.
20. Use mounting medium to mount the coverslip onto a microscope slide. Invert the slide onto a paper towel and press gently but firmly to remove excess mounting medium from under the coverslip.
21. Seal the coverslip with clear nail polish and let dry thoroughly before viewing under a microscope.
22. Image and store the slide at −20 °C. When combining RNA FISH with DNA FISH, RNA FISH-stained Samples must be imaged first and the coordinates of the cells on coverslip recorded. Following DNA FISH, the same cells should be imaged, to allow for analysis of overlaid images.

³⁰Denaturation time and temperature are very sample dependent and need to be optimized. Different cell types we routinely use vary from 5 min at 90 °C, 11 min at 80 °C, and 11 min at 95 °C.

3.10 Allele-Specific RNA FISH: Sample Hybridization

1. Start with permeabilized and fixed Samples plated on coverslips in 70% ethanol (*see* Subheadings 3.1 or 3.2).
2. Aspirate the ethanol using a vacuum apparatus and dehydrate the cells through an ethanol series (85%, 95%, and 100%) for 2 min each at room temperature.
3. Remove the coverslips from the well and air-dry at room temperature for 15 min (*see* Note 29).
4. While the coverslips are drying, prepare the allele-specific probe mix. In a 1.5 ml tube, add 1 μ l of probe mix for the reference allele, 1 μ l of probe mix for the alternate allele, and 1 μ l mask probe mix per 50 μ l guide probe.
5. Set up FISH hybridization. Pipette the probe onto parafilm-wrapped glass plate (*see* Note 4), invert the coverslip onto the droplet of probe and tap lightly with forceps to help the probe spread across the coverslip. Use 12 μ l of probe for coverslips of cells and 25 μ l of probe for coverslips with embryos. Use clear nail polish to seal around the edge of the coverslips to prevent drying and loss of embryos (*see* Note 31).
6. Incubate coverslips overnight at 37 °C in a humid chamber (*see* Note 5).
7. Gently remove nail polish using 70% EtOH. Transfer coverslips, sample side up, into a 6-well dish containing 2 ml pre-warmed 10% Formamide/2 $\times$ SSC using forceps.
8. Wash at 37 °C for 30 min in the dark (*see* Note 32).
9. Aspirate off the wash buffer and wash in pre-warmed 10% Formamide/2 $\times$ SSC with a 1:100,000–1:200,000 dilution of DAPI at 37 °C for 30 min in the dark (*see* Notes 18 and 32).
10. Aspirate the wash buffer and wash with pre-warmed 2 $\times$ SSC for 5 min at room temperature.
11. Use mounting medium to mount the coverslip onto a microscope slide. Invert the slide onto a paper towel and press gently but firmly to remove excess mounting medium from under the coverslip. Seal coverslips using clear nail polish.
12. Allow coverslips to dry in the dark.
13. Store at –20 °C.

³¹ Allele-specific probes diluted in the allele-specific hybridization buffer desiccate more quickly than the non-allele specific probe hybridization buffer. Thus, the coverslips should be sealed during allele-specific RNA FISH hybridization to prevent evaporation. Hybridization buffer evaporation results in the glass coverslips sticking to the parafilm and may cause loss of cells or embryos on the coverslip. This difference in hybridization conditions is likely due to the reduced concentration of deionized formamide in the allele-specific FISH hybridization buffer.

³² Cover the 6-well dish in aluminum foil.

Acknowledgments

This work was funded by NIH National Research Service Awards 5-T32-GM07544 (University of Michigan Predoctoral Genetics Training Program to M.C. and M.T.); a T32-HD079342 (University of Michigan Predoctoral Career Training in the Reproductive Sciences Program to M.C.); an NIH Director's New Innovator award (DP2-OD-008646-01), an NIH NIGMS R01 award (R01GM124571), and an NIH NICHD R01 award (R01HD095463) (to S.K.); and the University of Michigan Endowment for Basic Sciences (to S.K.).

References

1. Lee JT, Bartolomei MS (2013) X-inactivation, imprinting, and long noncoding RNAs in health and disease. *Cell* 152(6):1308–1323 [PubMed: 23498939]
2. Brockdorff N (2013) Noncoding RNA and Polycomb recruitment. *RNA* 19(4):429–442 [PubMed: 23431328]
3. Brown JD, Mitchell SE, O'Neill RJ (2012) Making a long story short: noncoding RNAs and chromosome change. *Heredity* 108(1):42–49 [PubMed: 22072070]
4. Maclary E et al. (2013) Long noncoding RNAs in the X-inactivation center. *Chromosome Res* 21(6–7):601–614
5. Maclary E et al. (2014) Differentiation-dependent requirement of Tsix long non-coding RNA in imprinted X-chromosome inactivation. *Nat Commun* 5:4209 [PubMed: 24979243]
6. Harris C et al. (2019) Conversion of random X-inactivation to imprinted X-inactivation by maternal PRC2. *elife* 8:e44258 [PubMed: 30938678]
7. Levesque MJ et al. (2013) Visualizing SNVs to quantify allele-specific expression in single cells. *Nat Methods* 10(9):865–867 [PubMed: 23913259]