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A Multifaceted FISH Approach to Study Endogenous RNAs and DNAs within Native Nuclear and Cell Structure

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Abstract

Fluorescence *in situ* hybridization (FISH) is not a singular technique, but a battery of powerful and versatile tools to examine the distribution of *endogenous* genes and RNAs in precise context with each other and in relation to specific proteins or cell structures. This unit offers the details of highly sensitive and successful protocols that were initially developed largely in our lab and honed over a number of years. Our emphasis is on analysis of nuclear RNAs and DNA to address specific biological questions about nuclear structure, pre-mRNA metabolism or the role of non-coding RNAs, although cytoplasmic RNA detection is also provided and generally discussed. Multi-faceted molecular cytological approaches bring precise resolution and sensitive multi-color detection to illuminate the organization and functional roles of endogenous genes and their RNAs within the native structure of fixed cells. Solutions to several common technical pitfalls are discussed, as are cautions regarding the judicious use of digital imaging and the rigors of analyzing and interpreting complex molecular cytological results.

Keywords

FISH; fluorescence in situ hybridization; nuclear structure; DNA; RNA; immunofluorescence; cytogenetics; histochemistry; chromosomes

INTRODUCTION

Over many years, *in situ* hybridization techniques have been developed into a battery of powerful methods whereby specific DNA and/or RNA sequences and proteins can be visualized directly within the context of cell and nuclear structure ((Langer et al., 1981; Langer-Safer et al., 1982; Johnson et al., 1991; Tam et al., 2002). This has had a transformative effect on cell and molecular biology, by bridging these two realms. The important insights this approach has brought into basic cell structure and function, genomic and epi-genomic organization, and novel roles of non-coding RNAs, are countless. This contribution to Current Protocols will focus on RNA detection with high resolution and sensitivity, particularly for nuclear RNAs. However, essential to many fundamental investigations is the ability to detect RNAs in direct relation to the genes from which they emanate, or the proteinacious nuclear structures with which they associate. Therefore this unit will cover RNA detection in the context of DNA, protein, and nuclear/cell structure, and will begin with illustrations of several types of technical analyses that address a variety of biological problems.

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As illustrated in Figure 1, fluorescent *in situ* hybridization (FISH) to DNA can detect not only abundant signals such as a chromosome library but as little as a few Kb of a single copy gene. However, this FISH DNA approach has been greatly extended to allow for the sensitive analysis of RNAs as well. The most basic and frequent use of RNA *in situ* hybridization is to evaluate the expression of a given gene at a single cell level, since other standard molecular methods rely on extraction of RNA from a cell population, and thus provide only information on *average* expression within that population. As illustrated for muscle-specific mRNAs in Figure 2, fluorescence *in situ* hybridization cannot only identify which cells are expressing RNA, but to illuminate the distribution of that RNA within the single cell. Protocols detailed here can visualize cytoplasmic and nuclear RNAs within the same cell; however, the optimal conditions for nuclear RNA, our emphasis here, may be distinct from that for cytoplasmic RNAs which vary depending upon the cell type and RNA studied. This is explained further below. Figure 2 also illustrates that comparison of two RNAs within the same cell provides insight into their relative abundance, providing probe sizes and labeling conditions are similar. In fact, it is often the case that the most sensitive detection for widely dispersed or low level mRNAs will be the focal nuclear RNA signal at or near the site of transcription, typically the highest concentration of a transcript in the cell.

Another important capability is to visualize RNAs in relation to specific protein structures using antibodies to endogenous proteins (or GFP-tagged proteins), particularly proteins that define specific sub-compartments of nuclear (or cytoplasmic) structure, as illustrated in Figure 3. In addition, oligonucleotide or other probes can be used to detect a larger class of RNAs, such as poly RNA. The power of molecular cytology is further enhanced by developments in digital imaging microscopy, covered in more depth in other units. As exemplified in Figure 4, digital imaging can quantify both intensity and spatial relationships between signals with much precision. An important earlier development was dual-and triple bandpass filters (Johnson et al., 1991) which allows multiple colors to be visualized through the microscope in precise spatial register, which is key for many biological questions, as further illustrated below.

In some cases it is desirable to visualize two regions of an RNA in precise relation, for example using specific intron versus cDNA probes to identify spliced versus unspliced transcripts. This is illustrated in Figure 5 for *XIST* RNA which is a large nuclear non-coding RNA that undergoes splicing, but the functional transcripts structurally associate with the interphase chromosome to induce its silencing. By hybridization to intronic versus exonic RNA it was determined that stable transcripts that accumulate are already spliced (Clemson et al., 1996). Similar approaches have provided insight into other aspects of pre-mRNA metabolism in relation to structure (Smith et al., 1999; Johnson et al., 2000; Smith et al., 2007). A technically more sophisticated matter is to visualize the precise distribution of an RNA emanating from that specific gene by simultaneous multi-color analysis of DNA and RNA. In this case it is necessary to hybridize sequentially to detect the RNA first in one color and then the DNA in a different color as illustrated in Figure 6 (and described in Basic Protocol 5). Detailed localization of this sort has shown that RNAs often accumulate *adjacent* to the gene, for example within specific domains of RNA metabolic factors (Figure 7A). Figure 7B illustrates that a triple labeling approach can provide remarkably precise visualization of the relationship of a given gene, its RNA, and a proteinaceous nuclear body (the Cajal Body) within a single cell (Smith et al., 2000).

Finally as genomics-based studies uncover the rich complexity of the human transcriptome, and the versatility of new types of large non-coding RNAs to form cytological structures or regulate chromatin, the ability to visualize novel RNAs within cells is proving critical to understanding their distinct biological roles, as illustrated by Neat I RNAs' architectural role in paraspeckles (e.g. Clemson et al., 2009). Another example, *XIST* RNA was found to

structurally associate with the whole domain of the inactive X chromosome territory in interphase nuclei, but in some cases its structural association on the chromosome can be captured at metaphase (Hall et al., 2009) (Figure 8). In addition, we developed a means to examine the overall distribution of hnRNA expression within nuclear structure, a convenient tool based on hybridization to RNA from the broad interspersed repeats present throughout the genome (Cot-1 DNA) which will be present in intronic containing pre-mRNAs. This, now well established approach, clearly discriminates the inactive from the active X chromosome (Hall et al., 2002), but also the zones of heterochromatin that typically encircle the nuclear and nucleolar peripheries (Tam et al., 2004, Figure 9).

Experiments such as these require methods with high sensitivity, specificity and resolution, while being versatile enough to preserve and detect multiple RNA/DNA/protein components simultaneously in the same nucleus. Although cytoplasmic mRNA can also be detected using these methods, our focus will be on the challenge of detecting often very small quantities of nuclear transcripts, and will highlight the greatest value of this technique: the visualization of *endogenous* RNAs expressed from their *native* location and preserved in their natural nuclear environment. Approaches to follow labeled DNA transgenes or RNAs in living cells provide another exciting dimension (Hu et al., 2009). Understanding many key aspects of cell structure and nuclear genome organization demands analysis of native genes and RNAs in their natural structural context.

BASIC PROTOCOL 1 – CELL PREPARATIONS – DETERGENT EXTRACTION PRIOR TO FIXATION

When choosing a cell preparation protocol it is important to consider the aim of the experiment, the RNA sequence to be targeted, and the cell type to be used. These protocols are designed to optimize nuclear RNA detection but in most cases will also detect cytoplasmic RNAs at the same time. (e.g., Figures 2 and 3). The main distinction between nuclear and cytoplasmic RNA detection protocols concern cell extraction. Although some cells require no extraction (e.g., lymphoblast cells, Lawrence et al., 1989) most cell types require the nucleus be permeabilized by detergent extraction prior to fixation to allow the probe to penetrate the nucleus. Early comparisons of RNA detection with and without any detergent based extractions showed that nuclear RNAs did not appear significantly diminished or disrupted with this methodology (Carter et al., 1991; Xing & Lawrence, 1991; Clemson et al., 1996). Cells that have extensive cytoplasmic areas, such as cultured skeletal muscle, need longer detergent extraction prior to fixation to penetrate the nucleus. However, extraction procedures must be vigorously controlled because it is a delicate balancing act between sufficient permeabilization and preservation of structures or labile RNA. Once an unfixed cell is permeabilized, nucleases and proteases are released, so minimizing the time the cells are permeabilized before fixation is key. Cytoplasmic mRNA is more vulnerable to being lost during longer extractions (1-5 minutes), so limiting extraction to 30 seconds, or extracting *after* fixation is recommended (as described in Alternate Protocol 1).

The Basic Protocol 1 below has been optimized for monolayer cells with large cytoplasms, while the Alternate Protocol 1 is best for cells with less cytoplasm (e.g. ES cells and lymphoblasts). For storage of fixed cell samples 70% ethanol storage is fine for RNA (or DNA), but some protein immuno-detections work better if cells are stored in 1xPBS. Ethanol storage is preferred due to the longer preservation of the sample (several months), while 1xPBS is only good for a week or two. It is recommended to test these various conditions to optimize detection with your reagents. Sub-confluent cell density is preferred for cell adherence and penetration.

Materials

Glass coverslips (22×22 mm is optimal). Non-adherent cells can be attached to glass coverslips using Cell-Tak (BD Bioscience).

Coplin jars for 22×22 mm coverslips (Thomas Scientific)

Ice bucket

Ice

Fine tip forceps

Petri dishes

Hanks Balanced Salt Solution (HBSS)

Cytoskeletal buffer (CSK)

200mM Vanadyl Ribonucleoside Complex (VRC)

Triton X-100 10% solution (Roche)

4% (v/v) Paraformaldehyde (Ted Pella) in 1x PBS (PFA)

70% (v/v) Ethanol

1xPBS

Sample Processing

1. Rinse coverslips with monolayer cells twice in room temperature HBSS to remove media.
2. Transfer coverslips from HBSS to a Coplin jar, on ice, filled with 10 ml CSK buffer. This is a quick rinse; coverslips are in the cold CSK buffer for less than a minute.

Extraction

3. Transfer coverslips to another coplin jar, on ice, with CSK buffer, 0.5% Triton X-100 for extraction, and 10 mM VRC for RNA preservation. Cells can be extracted in this buffer anywhere between 30 seconds to 5 minutes

   i. 30 seconds for cytoplasmic RNAs and cells lacking substantial cytoplasmic structure (i.e., hESC)

   ii. 3 minutes for fibroblasts

   iii. 5 minutes for more structured cells (e.g., multinucleated myotubes)

Fixation

4. Transfer coverslips to a third Coplin jar containing 4% PFA at room temperature. Incubate cells for 10 minutes.

5. Store coverslips in 70% ETOH or 1×PBS at 4°C until ready to use.

ALTERNATE PROTOCOL 1 – DETERGENT EXTRACTION AFTER FIXATION

This method is used for extracting cells that lack substantial cytoplasm or when trying to detect cytoplasmic RNA or proteins that are sensitive to extraction.

Materials

See Basic Protocol 1

Sample Processing

1. Rinse coverslips twice in room temperature HBSS to remove media.

Fixation

2. Transfer coverslips to a Coplin jar containing 4% PFA at room temperature. Incubate for 10 minutes.

Extraction

3. Transfer coverslips to a Coplin jar, on ice, with CSK buffer, 0.5% Triton X-100 for extraction, and 10 mM VRC for RNA preservation. Incubate anywhere from 30 seconds to 5 minutes on ice.

4. Transfer coverslips to a Coplin jar, on ice, with 10 ml CSK buffer to rinse off the triton.

5. Coverslips are stored in 70% ETOH or 1×PBS at 4°C until ready to use.

BASIC PROTOCOL 2 – PREPARATION OF PROBES FOR IN SITU HYBRIDIZATION BY NICK-TRANSLATION

Choice of Probe

The success of an *in situ* hybridization is largely contingent upon an appropriate choice of DNA probe sequence. The decision to use a genomic or cDNA sequence, as well as the length of the sequence, will impact the intensity of the hybridization signal. For detection of a DNA locus or unspliced primary transcripts, a larger genomic sequence with its many introns, exons and flanking sequences makes for a more efficient hybridization. The choice of probe is more complicated for nuclear RNAs, because they contain exons and the choice of probe depends on the question asked (intron specific, exon specific, genomic or cDNA). However, it is beneficial to use a cDNA probe for detection of cytoplasmic mRNA. Our lab can detect gene DNA signals with probes that target sequences as short as 1-2 Kb in interphase nuclei and metaphase chromosomes, although this requires all conditions to be optimal. Oligonucleotides as small as 20-25 bp have also been used to detect certain nuclear RNAs (Smith and Lawrence, 2000), however it is best to use sequences that target 5-10 Kb *or more* for single copy gene detection. Cosmid or BAC probes yield strong signals, and detect with high efficiency (>90%). However, it is necessary to ensure that these large sequence probes have been fragmented to sufficiently small sizes in the nick-translation process to prevent high background (see details in Nick-Translation below). The relatively high probe concentrations used to detect single gene transcripts can result in higher non-specific background, and this is more pronounced for longer probe fragments which can self-associate and form aggregates on the slide during hybridization. When using cloned plasmid DNA as a probe, it is not necessary to remove the inserted sequences from the rest of the vector sequence. Similarly, the presence of repetitive sequences is also not an issue provided that sufficient Cot-1 DNA is added as competitor.

The Cot-1 DNA fraction represents the repetitive portion of the genome which re-anneals the fastest following fractionation and denaturation of the genome. This repetitive DNA pool is routinely used as an unlabelled competitor in hybridization experiments to reduce binding of probes to non-specific loci. However, several years ago we developed the use of Cot-1 DNA as a labeled probe to broadly survey hnRNA containing intronic and other repeats throughout nuclei (Hall et al., 2002; Clemson et al., 2006). As discussed in the introduction and shown in Figure 9, this can be used to discriminate regions of heterochromatin versus euchromatin.

Nick-Translation

In situ probes used in our lab and many others are generally double stranded DNA, most typically labeled by nick-translation, often to incorporate a digoxigenin-11-dUTP or biotin-16-dUTP nucleotide, however a variety of other labels are now available. Post nick-translation, the fragment size should be roughly 200 bp and the range of fragment sizes should not exceed 700-800 bp (to reduce the occurrence of background). Fragment size can be adjusted by varying the concentration of nick-translation enzymes, but the availability of commercial and quality controlled nick-translation enzymes has eliminated the need for too much adjusting. Basic Protocol 2 utilizes the nick-translation enzyme mix from Roche.

Materials

1 μg of *clean* DNA

50 nmol Biotin-16-dUTP or 25 nmol Digoxigenin-11-dUTP (Roche)

Nick-translation mix (Roche)

Nucleotide mix

Nuclease free water

0.5 M EDTA

5% Sodium Dodecyl Sulfate (w/v) (SDS)

Deoxyribonucleic acid, single stranded, from salmon testes (Salmon Sperm DNA)

(Sigma) and E.coli tRNA (Roche) (ssDNA+tRNA)

3 M Sodium acetate

70% + 95% Ethanol (v/v) (4°C or colder)

15°C Water bath

80°C Heat block

Microcentrifuge

1. In a microcentrifuge tube add 4 μl of the nucleotide mix, 1 μg of DNA, 6 μl of either biotin-16-dUTP or digoxigenin-11-dUTP, and water to bring the volume up to 16 μl. Mix and briefly spin to pool the solution.
2. Add 4 μl of Nick-translation mix, gently stirring with pipette tip to mix into the solution.
3. Incubate for 2 1/2 hours in a 16°C water bath.

4. Stop enzyme reaction by adding 2 μl of 0.5 M EDTA and 2 μl of 5% SDS and heat on an 80°C heat block for 10 minutes.
5. Remove any unincorporated nucleotides by ethanol precipitation. Add 2 μl ssDNA + tRNA, 2.6 μl (0.1× the volume) 3M sodium acetate, 71.5 μl (2.5× the volume) cold 95% ethanol. Incubate at −20°C for a minimum of 1 hour or overnight.
6. Pellet precipitated DNA by spinning at 10,000 rpms for 30 minutes.
7. Pour off supernatant into a kimwipe, while making certain the pelleted DNA stays in place. Rinse DNA with 500-1000 μl cold 70% ethanol, by gently inverting the tube several times; again make certain the DNA pellet does not become detached.
8. Pour off 70% ethanol in to a kimwipe and air dry.

   Drying of the DNA can be hastened by briefly spinning in a speed-vac lyophilizer. However, over drying can make it difficult to get the DNA back into solution.
9. Resuspend probe with 100 μl of nuclease-free H_20 and store at −20°C.

BASIC PROTOCOL 3 - HYBRIDIZATION TO RNA

Reagents used for hybridization should be free of any RNase and be of high molecular grade quality. For convenience, overnight incubations for hybridizations can be done, but three hour hybridization incubations are successful and could be beneficial to minimize any possible RNA degradation. As mentioned above, unlabeled Cot-1 DNA, in conjunction with other nonspecific nucleic acid competitors, is added in excess to the labeled DNA probe prior to hybridization to minimize cross-hybridization to highly repetitive sequences. Cot-1 DNA that is used for competition should be from the same species as the cell samples, as the Cot-1 DNA repetitive sequences are species specific. Probes labeled with the two different analogs can be hybridized and detected concurrently, in two distinct colors. Biotin and digoxigenin probes are indirectly detected by avidin or anti-digoxigenin antibodies that have been conjugated to different fluorochromes (see examples below). Double stranded DNA is not accessible for hybridization in this protocol because there is no DNA denaturation step.

Materials

50 ng Biotin or Digoxigenin labeled DNA probe

Human Cot-1 DNA (Roche) or Mouse Cot-1 DNA (Invitrogen)

Salmon Sperm DNA + E. Coli tRNA

100% ethanol (4°C or colder)

Formamide (Sigma)

Hybridization buffer

RNase inhibitor (RNasin-Promega or RNase OUT-Invitrogen)

Parafilm

4× SSC, 2× SSC, and 1× SSC

1% BSA in 4xSSC (w/v)

0.1%Triton X-100 (Roche) in 4xSSC (v/v)

Dylight 488 or 594 Streptavidin (Jackson Immuno Research) or Anti-digoxigenin

Fluorescein (Roche)

DAPI (Sigma)

Glass Micro Slides (Corning)

Vectashield (Vector Labs)

Nail polish (clear)

Slide folders

Hybridization

1. Prepare coverslips by dehydrating them in 100% cold ethanol for 10 minutes and air dry.
2. Aliquot and lyophilize 50 ng (5 μl) of labeled probe, 10 μg Cot-1 DNA, 10 μg each of ssDNA+ tRNA.
3. Resuspend the lyophilized probe with 10 μl of 100% formamide.
4. Denature probe for 10 minutes on an 80°C heat block.
5. Add 10 μl of RNA hybridization buffer with 80 U/μl of RNasin.
6. On a glass plate lined with a piece of parafilm spot the probe/ hybridization buffer mixture directly onto the parafilm (Figure 10). With forceps, place coverslip, cell side down, on top of the probe gently pressing down on the coverslip to allow the probe to spread across the slip. Avoid pressing down too hard or too much, or the probe will come out under the edges of the coverslip leaving less probe to hybridize.
7. Cover all coverslips with another piece of parafilm and seal around all of the edges to prevent the probe from drying out (Figure 10).
8. Incubate in a humid chamber at 37°C for 3 hours to overnight.

Washes

9. Wash coverslips for about 20 minutes each in 50% formamide in 2× SSC at 37°C, 2× SSC at 37°C, 1× SSC at room temperature on a shaker and finally in 4× SSC at room temperature for about 2 minutes to equilibrate cells before detection.

Detection

10. Dilute secondary antibodies 1.5-2 μg/ml into 500 μl of 4× SSC + 1% BSA.

11. Incubate coverslips with 80 μl of diluted secondary sealed in parafilm (as in Figure 10) in a 37°C humid chamber for 1 hour.

Rinses

12. Rinse coverlips for 10 minutes each in 4× SSC, 4× SSC+0.1% triton X-100, 4× SSC on a shaker at room temperature (in the dark).

13. Counterstain cells with DAPI (about 1 minute), rinsing twice with 1× PBS.

14. Mount coverslips to glass slides (cell side down) with 1 drop of vectashield anti-fade medium and seal around the edges of the coverslip with nail polish.

15. Slides are stored at −20°C in dark slide folders, and can last for weeks, months and in some cases years.

ALTERNATE PROTOCOL 3 – HYBRIDIZATION TO DNA

The above RNA hybridization method detects single-stranded nucleic acids. The probe does not hybridize to DNA, because the DNA was not denatured. However, hybridization to cellular DNA can also detect RNA sequences from an active gene if RNA is not eliminated prior to hybridization. RNA can be removed by NaOH hydrolysis, pre-hybridization treatment with RNase A, or, where useful, a post-hybridization treatment with RNase H. NaOH treatment has the added benefit of simultaneously denaturing cellular DNA for the following hybridization. In some cases we couple NaOH treatment and heat denaturation because NaOH is not as consistent a denaturing method as heat. To validate whether the signal is DNA as opposed to RNA, negative controls include RNA removal by RNAse, use of cells that do not express the gene, or incubation in transcriptional inhibitors (e.g. actinomycin D) prior to fixation. If RNA has been completely eliminated, these should be completely negative. Note, treatment with RNAse or NaOH does not always eliminate all RNA signals, although it is often assumed it does, therefore a good negative control is to omit DNA denaturation, *including NaOH RNA removal*, as NaOH denatures the DNA.

Materials

Water bath pre-warmed to 80°C

Coplin jar pre-warmed to 80°C in the water bath

10-20 ml beaker

Microwave

Thermometer

70% Ethanol (v/v)(4°C or colder)

70% Formamide (Sigma) in 2× SSC (v/v) (made fresh)

In addition to all materials listed in Basic Protocol 3 (except for RNase inhibitor)

Hydrolysis of RNA

1. Transfer coverslips from 70% ETOH storage (4°C) into Coplin jar with 0.2N NaOH, incubate 5 minutes at room temperature. (This step can be omitted if you don’t need to get rid of all of the RNA).
2. Rinse cells with 70% ETOH.

Heat Denaturation of Cellular DNA (as needed)—This step must be done in a chemical fume hood so as not to breathe in formamide fumes.

3. Microwave 70% formamide in 2× SSC in beaker on high 9-10 seconds or until the temperature of the formamide is 75°C-80°C (try not to boil over, time will vary with microwave).

4. Pour hot formamide into the pre-warmed Coplin jar.

Coplin jars have been known to crack when hot formamide is added to a room temperature jar.

5. Transfer coverslips into jar and incubate 2 minutes.

Temperature of the formamide may decrease with the addition of many coverslips.

6. Transfer coverslips to cold 70% ethanol. Incubate for 5 minutes.

7. Replace 70% ethanol with cold 100% ETOH. Incubate for 5 minutes.

8. Air dry coverlips.

DNA Hybridization—See Basic Protocol 3 – RNA Hybridization. RNase inhibitor is omitted from the hybridization buffer, but all steps are the same.

BASIC PROTOCOL 4 – RNA OR DNA HYBRIDIZATIONS WITH OLIGONUCLEOTIDE PROBES

Oligos can be used for some DNA and RNA detection purposes as well. To increase signal strength, you can also pool oligos made to tandem sequences, although for some experiments we have had success detecting RNA with single oligos as small as 20-25 nt (3′ biotin labeled) (Smith et al., 2000). Oligos can be purchased with a variety of end labels, and sensitive LNA (locked nucleic acid) oligos are becoming more available. Because of the differences in hybridization properties of LNA oligos which bind very stably (Jepsen et al., 2004), care should be taken to adjust formamide to increase stringency as needed to avoid less specific binding.

When hybridizing with an oligo probe the proper conditions for stringency are based on the sequence content. Oligos with a high AT content are less stable and therefore need a lower concentration of formamide than ones with a higher GC content. Generally our lab uses 15% formamide as a good starting point when trying new oligo probes (detailed below), but the range is within 10-25%, and rarely is 50% used. The same concentration of formamide used to hybridize the oligo is used for the post-hybridization washes. The specificity of the oligo sequence eliminates the need for addition of Cot-1 DNA (as cold competitor), and the lower formamide concentrations eliminate the need to lyophilize the probe before the hybridization step. In addition, denaturation of the single-stranded oligo probe is not necessary, although oligos can be heat denatured without any negative consequences (and this may even be advantageous for a longer oligo that could fold and anneal to itself).

Materials

See Basic Protocol 3 (Hybridization to RNA) or Alternate Protocol 3 (Hybridization to DNA)

Hybridization

1. Prepare cells as described above for either RNA or DNA detection.
2. Mix 5 picomoles of oligo probe into 15% formamide, final volume of 10 μl.
3. If desired, denature probe on 80°C heat block for 10 minutes.
4. Follow steps 5-8 of Protocol 3.

   RNase inhibitor from hybridization buffer can be omitted if detecting DNA.

5. Wash coverslips for 20 minutes each in 15% formamide in 2× SSC at 37°c, 2× SSC at 37°c, 1× SSC at room temperature on a shaker and 4× SSC at 37°C for about 2 minutes to equilibrate cells before detection.

Detection

6. Follow steps 10-15 of Basic Protocol 3.

BASIC PROTOCOL 5 – HYBRIDIZATION TO RNA AND DNA SIMULTANEOUSLY IN TWO DISTINCT COLORS

The use of multiple fluorometric labels has numerous applications to study spatial interactions amongst various cellular components. Our lab has modified our FISH protocols to allow the visualization of RNA in one color and the corresponding DNA in another by using differentially labeled probes *for the same sequence*. In this way, individual genes can be distinguished from their transcripts in the same nucleus, which is valuable for studies examining spatial organization during RNA metabolism. For example, this allowed us to discern that transcript foci from some genes are post transcriptional accumulations that emanate vectorially away from their sites of transcription (Figure 6), and where some viral RNA transcripts appear as long “tracks”. For RNA/DNA sequential hybridization, the two probes are hybridized sequentially following Basic Protocol 3 (hybridization to RNA). The RNA signal is fixed within the nucleus before the RNA is eliminated with NaOH to ensure that the second hybridization detects DNA and not lingering RNA.

Materials

See Basic Protocol 3 (Hybridization to RNA) and Alternate Protocol 3 (Hybridization to DNA)

1. Hybridize and detect RNA first following Basic Protocol 3, using either a biotin or digoxigenin labeled probe and the corresponding detector (avidin or anti-dig).
2. Incubate the cells in 4% PFA for 10 minutes at room temperature to fix the RNA detector signal. It is not necessary to visualize the samples under a microscope before moving forward with the DNA hybridization.
3. Rinse twice briefly in 1× PBS.
4. NaOH treat to hydrolyze the RNA, heat denature and hybridize to the DNA using a probe that is labeled differently than the RNA probe, following Alternate Protocol 3.

BASIC PROTOCOL 6 – PROTEIN DETECTION WITH HYBRIDIZATION TO RNA OR DNA

Like dual label RNA-DNA hybridizations, protein immunofluorescence combined with hybridizations can be an extremely valuable tool in studying whether genes or transcripts associate with particular nuclear structures or compartments (Figure 3). Double and triple label experiments can be achieved with the use of two to three distinctive fluorophores, including but not limited to, FITC, Dylight 488 and 594, along with an Aminomethylcoumarin-conjugated secondary antibody. We often use the coumarin derivative, aminomethylcoumarin (AMCA) as our third fluorochrome, which is captured by the same filter set as DAPI stain. However, due to its low coefficient of extinction, it is not bright enough to label transcripts or gene signals, so we only use this to label abundant proteins (Figure 7). In multi-label experiments, the hybridization and immunodetection are

often done sequentially and are fixed in between each of the steps to preserve the signal before detecting another.

Some protein epitopes can be harmed by a prior hybridization and in those cases it is advisable to do the immunostaining before the RNA or DNA hybridization. If RNA hybridization is to follow the antibody staining, the addition of an RNase inhibitor (such as RNasin, Promega) is recommended to preserve the RNA in case the antibody contains any RNases. Do not use VRC, although it is suitable for the preservation of RNA during cellular extraction we have found that when included in the antibody reagents it can adversely affect the outcome of some protein detections.

Materials

Primary antibody of interest

1% BSA in 1× PBS

1× PBS

0.1% Triton X-100 in 1× PBS

Fluorescent-conjugated secondary antibody

4% Paraformaldehyde in 1× PBS

Coplin jar

Glass plate

Parafilm

37°C humid incubator

Primary Antibody

1. Rinse coverslips in 1xPBS 10 minutes
2. Dilute antibody in 1% BSA in 1× PBS (dilution is antibody dependent)
3. On a glass plate lined with a piece of parafilm spot 20-80 μl of the diluted antibody directly onto the parafilm. With forceps, place coverslip, cell side down, on top of the antibody (Figure 10).
4. Cover with 2nd sheet of parafilm, seal the sides like an envelope to prevent evaporation and incubate 1 hr at 37°C.

Washes

5. Wash coverlips in a Coplin jar on a shaker for 10 minutes each: 1× PBS, 0.1% Triton X-100 in 1× PBS, and 1× PBS

Detection

6. Dilute fluorescent-conjugated secondary antibody 1× PBS+ 0.1% Triton X-100 to a concentration of about 3 μg/ml.

7. On a glass plate lined with a piece of parafilm spot 20-80 μl of the diluted antibody directly onto the parafilm. With forceps, place coverslip, cell side down, on top of the antibody.

8. Cover with 2nd sheet of parafilm, seal the sides like an envelope to prevent evaporation and incubate 1 hr at 37°C.

9. Repeat washes as in step 5.

10. Fix protein signal for 10 minutes in 4% paraformaldehyde, PBS.

11. Rinse coverslips twice with 1× PBS.

12. Proceed with Basic Protocol 3 or 4.

Reagents and Solutions

10 x PBS (Phosphate Buffered Saline)

4 liters	
320 g	NaCl
8 g	KCl
8 g	KH_2PO_4
46 g	Na_2HPO_4
pH to 7.4	

20 x SSC (Saline Sodium Citrate)

4 liters	
696 g	NaCl
352.8 g	NaCitrate
pH to 7.4	

Cytoskeletal Buffer (CSK)

100 mM	NaCl
300 mM	Sucrose
10 mM	PIPES, pH 6.8
3 mM	$MgCl_2$

Store at 4°C for 1 month

DAPI—100 μg/ml DAPI (Sigma) in 1× PBS

Stored at 4°C in the dark for a few months

Hybridization Buffer—2 parts - 50% (w/v) Dextran Sulfate, autoclaved

1 part - Bovine Serum Albumin (BSA) (Roche)

1 part - 20× SSC

1 part - Nuclease Free Water

Store at 4°C for three months

Nucleotide Mix—600 μM each of dATP, dCTP, and dGTP (Roche)

Store at −20° for several years

Salmon Sperm DNA (Deoxyribonucleic acid, single stranded, from salmon testes; Sigma) + *E. Coli* tRNA (Roche)—Add 10 mg of *E.Coli* tRNA to the tube of 10mg/ml Salmon sperm DNA

Mix well

Store at −20°C for several years

Vanadyl Ribonucleoside Complex (Sigma)—Thaw and aliquot 500 μl volumes on ice

Store at −20°C for several months

Commentary

Background Information—Many laboratories have contributed to the development and application of cytological hybridization over a span of several decades. In 1969 Pardue and Gall first hybridized radioactive DNA to abundant satellite sequences directly within cells, using autoradiographic methods to detect and localize specific DNA sequences *in situ*. This was a major milestone. However, due to the limited resolution and sensitivity of autoradiography, it was not possible to visualize within an individual cell or nucleus the location of a single gene or cognate primary transcript using isotopic labeling. To circumvent these limitations, multiple labs worked to develop non-isotopic *in situ* hybridization methods; for example, the notable work on biotin labeling of nucleic acids from the David Ward lab (Langer et al., 1981 and Langer-Safer et al., 1982). Initially the biotin labeling approach successfully provided increased resolution for very abundant sequences, such as whole chromosome libraries (Figure 1) (Pinkel, 1988, Lichter, 1992), but lacked sensitivity for single genes. However it was subsequently shown that this limited sensitivity was largely due to the *in situ* hybridization protocols being used, not to an inherent limit in the detector sensitivity. A quantitative approach was applied to analyze different components of the process separately, to identify the more limiting factors. This uncovered a combination of weaknesses in many common protocols, including poor nucleic acid preservation (particularly RNA), hybridization conditions that limited *in situ* molecular interactions but generated background, and detection conditions that promoted sticking of fluorescent avidin reagents.

When these different aspects of the total process (preservation, hybridization and detection) were separately and quantitatively investigated to identify optimal procedures (Lawrence and Singer, 1985), non-isotopic detection with biotin proved to have sufficient sensitivity to detect not only abundant signals such as a chromosome library, but targets as small as a few Kb of a single copy gene or its cognate mRNA within a single cell (Lawrence et al., 1988; Lawrence et al., 1989; Xing et al., 1993). The examples summarized throughout this unit exemplify the continual development of molecular cytology and fluorescence microscopy to address an ever-expanding diversity of biological questions.

Critical Parameters

Fluorescence Microscopy: Fluorescence microscopy provides the advantage of high sensitivity, resolution, and multi-labeling to enable analysis of multiple nucleic acids or proteins within cell structures. In some cases it is powerful enough to detect relatively low

levels of single gene transcripts with probes targeting as little as 22 base pairs (Smith et al., 2000). However the sensitivity of the detection will be significantly influenced by the distribution of small RNAs with the cell, since very small dispersed sequences can be more difficult to discern above background.

Details of fluorescence microscopy are covered elsewhere (see UNIT 4.4). Results of the FISH procedures described here can be analyzed by confocal microscopy or by digital imaging and epi-fluorescence microscopy. The latter sometimes provides the advantage of less rapid bleaching of tiny fluorescence signals. Confocal microscopy is particularly advantageous for the 3-D analysis of thicker specimens like tissues or embryonic bodies, however 3-D analysis can also be done at single cell resolution using a series of z-stacks from a wide-field fluorescence microscope and applying a deconvolution algorithm which corrects for out of focus light. Such analyses should be done with high magnification and high numerical aperture lenses (a 100X, 1.4 NA oil immersion objective is recommended).

Analysis: An enormously important and complex aspect of molecular cytology is the analysis of results. There are many key considerations and potential pitfalls depending upon the nature of the technical and biological problem being addressed. Therefore, for anything other than the most straightforward questions, it is recommended that analysis of particular questions and results initially be done with the involvement or advice of someone who has substantial experience in the interpretation of molecular cytology results of a similar nature.

One common area of misconception concerns the scale of structures or distances between signals seen through the microscope, in relation to molecular distances. For example, because the size of most gene signals is at or below the level of resolution obtained by fluorescence microscopy (~0.2 microns or at best close to 0.1 microns with deconvolution), the actual dimension of the physical length of DNA is generally much smaller than it appears. Often if two signals appear close but separate by fluorescence microscopy, it is presumed that such proximity provides evidence of an interaction thought to be at a molecular scale (discussed in Lawrence and Clemson, 2008). This is not the case; signals that are visually separated through the microscope are located far apart on a molecular scale.

Another notable potential pitfall in analysis of molecular cytology results concerns the ease of digital imaging to manipulate images in such a way that the signals can appear brighter than they actually are, which inappropriately changes the relative intensity of two signals in different ways. Increasingly it is recognized that strict procedures should be followed in the processing of digital microscopy that preserves the information inherent in results as they appear through the microscope. In our own view, it is important for those interpreting results to do so based on analysis of many randomly selected cells directly through the microscope without any processing. Digital images acquired using CCD cameras offers easy and fast acquisition and storage, as well as flexible manipulation of image size and contrast. However, data must be recorded in a manner that accurately reflects the signals in the actual sample as validated by eye through the microscope (see Rossner and Yamada, 2004).

Finally, another potential pitfall that has resulted in misinterpretations in the literature regards the potential that very bright signals, particularly red signals, (e.g., rhodamine) can “bleed through” the fluorescein filter, in some cases leaving a weak green signal that appears to overlap the red signal. If such weak signals are misinterpreted, and even sometimes are imaged to appear stronger, substantial scientific misinformation regarding the interaction of specific molecules within cells can result.

For these reasons, we recommend that even where FISH procedures are successful, individuals who are not experienced in the analysis of these results seek the input of those

who are. Similarly, in our own laboratory key findings before publication need to be evaluated directly through the microscope by at least two individuals, preferably one of them being a principal investigator.

Troubleshooting—Some troubleshooting has been incorporated into the protocols above, however here we will pinpoint some specific issues and how to address and resolve them. *In situ* hybridization, particularly the more involved double and triple labeled experiments, requires having all of the right conditions for each experimental step in place. At times this can be tricky. The cells must be extracted, fixed and stored in a fashion that is suitable for all components of the experiment. The DNA sequence used as a probe must be sufficiently long, labeled and nicked into pieces that are not too large. Temperature and pH need to be considered during denaturation (where necessary) and hybridization. Once key parameters have been met, subsequent experiments should run more smoothly.

Problem: No RNA signal: Before doing an RNA hybridization (or if the first RNA hybridization was unsuccessful), test the probe in a DNA hybridization to insure that the probe is working. Chances are if the probe works for DNA it will work for RNA, provided the gene is active and there is sufficient RNA. Even when cytoplasmic RNA is present at a very low level, RNA is generally detectable at/near the site of the active gene. Where possible it is advisable to include a probe that is known to work as a positive control to test the preservation of the RNA.

Problem: No DNA signal: If there is no DNA signal, it is possible that the DNA was not successfully denatured. The denaturation solution must be at or slightly above 70°C for the duration of the step in order for the DNA to properly denature. The more samples processed together, the cooler the formamide solution becomes when the coverslips or slides are added to the heated formamide. The water bath used should be pre-heated to 75-80°C. A two minute denaturation is usually enough time to denature DNA, but sometimes an additional 30 seconds to 1 minute is needed. Once the denaturation solution (70% formamide in 2× SSC) is made (fresh for each experiment), the solution should be at a pH between 7-8.

Problem: No antibody signal: If there is no antibody detection for an expected protein, the first thing to consider is the fixation and storage of the cell samples. A number of protein epitopes do not react well with ethanol storage, therefore the antibody staining will not work. Cells should then be stored in 1× PBS. In addition, some proteins can be removed if the cells are extracted prior to fixation, therefore fixing the cells prior to extraction may be necessary. It is always good to test these conditions before coupling with a hybridization.

Problem: Loss of RNA Signal in RNA/DNA Sequential Hybridizations: Some RNA signal may be lost during the sequential DNA hybridization, but most should remain. To maintain the RNA signal, use an indirect method to detect the probe. An indirectly labeled probe is preferred because the secondary detector (e.g. avidin or anti-dig) is what is fixed by the paraformaldehyde prior to denaturation and DNA detection.

Problem: Protein with RNA or DNA hybridization – Antibody signal lost: Although it is occasionally preferable to do a protein antibody detection prior to a hybridization, some antibodies work better *after* a hybridization. Unlike RNA/DNA sequential hybridizations, >the order of protein/hybridization detection can be switched, and may make a difference. In addition, simply re-staining the protein with the primary and secondary antibodies can save an experiment where the initial protein detection was unsuccessful. Occasionally adding a little secondary antibody (used to detect the protein) into the RNA or DNA detection step also helps. However, keep in mind that this can increase background.

Problem: Background: Two common issues can contribute to high background in FISH. First, some cells have endogenous biotin, which will generate background with the avidin detector. Thus, a different choice of probe label (digoxigenin or direct fluorochrome) is needed. Second, large probe fragment sizes (greater than 700-800 bp) can create large punctuate background across the whole slide, because the probe is not able to penetrate the nucleus. Checking the probe size by running it on a gel will answer whether the probe is in the right size range. Lastly, incubation times for hybridizations and detections should not go beyond the times specified in these protocols as this too could cause unwanted background issues.

Time Considerations—The process of probe nick-translation, cell fixation, RNA hybridization (3 hour incubation) and detection can take roughly 11 hours at minimum. If a sequential DNA hybridization is included, add approximately 21 hours making a total 32 hours, where as antibody detections will add an additional 3 hours. To break up the amount of time dedicated to the whole process, some aspects can be divided and prepared on different days for convenience. Cells can be extracted, fixed, and stored at 4°C until ready for use, and the probe can be nick-translated several days, weeks, or months ahead and stored at −20°C reducing RNA hybridization time to roughly 6 hours.

Acknowledgments

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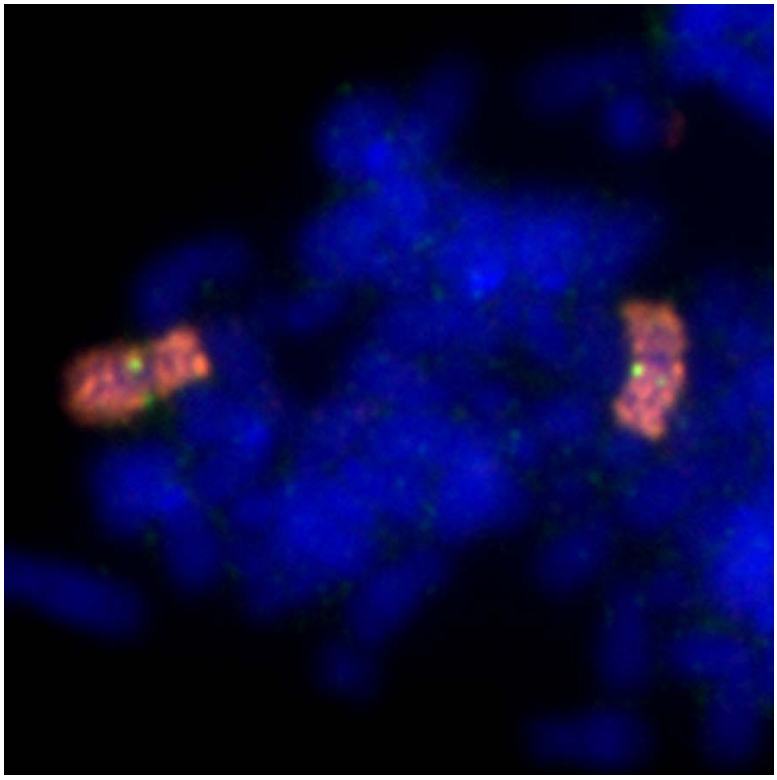

Figure 1. Single gene detection and chromosome painting

The X-linked *XIST* is detected in green with a 9kb genomic probe, while the entire X-chromosome is detected in red with a whole chromosome library which “paints” the chromosome.

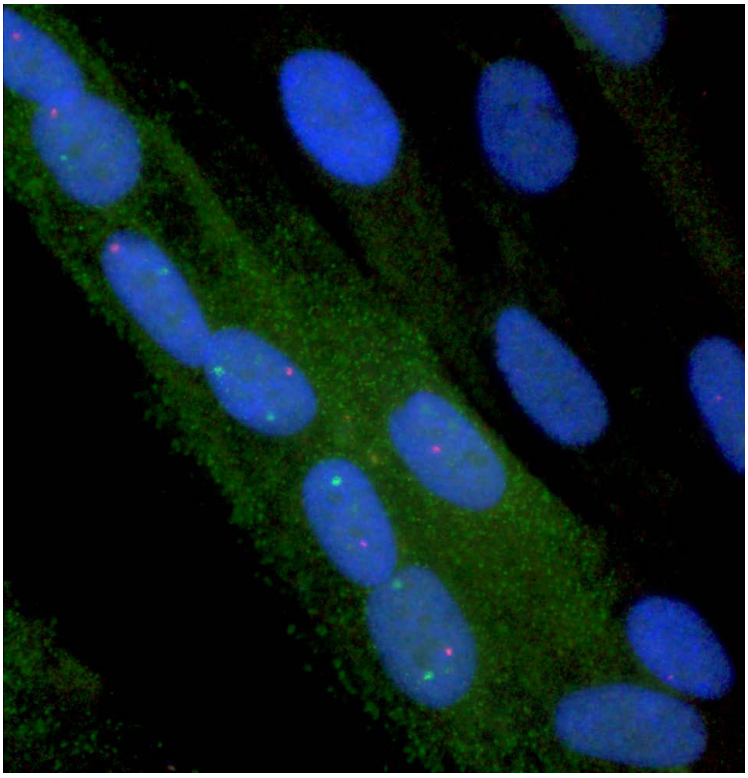

Figure 2. Cell-type specific gene expression of two muscle RNAs shows the distribution of nuclear and cytoplasmic mRNAs

Myosin heavy-chain (MyHC) (green) and dystrophin (red) are expressed in differentiated myotubes (the multinucleated cells at upper right), while undifferentiated myoblasts (single nuclei in bottom left) do not express these muscle specific genes (Smith et al., 1999. Originally published in J Cell Biol 144(4):617-29). The MyHC mRNA is abundant in the cytoplasm, whereas the extremely low level dystrophin mRNA is barely detected in the cytoplasm with this 10 kb genomic probe. Pre-mRNA associated with the gene loci is clearly detected, with one RNA focus for dystrophin from the one X-linked gene (male cells).

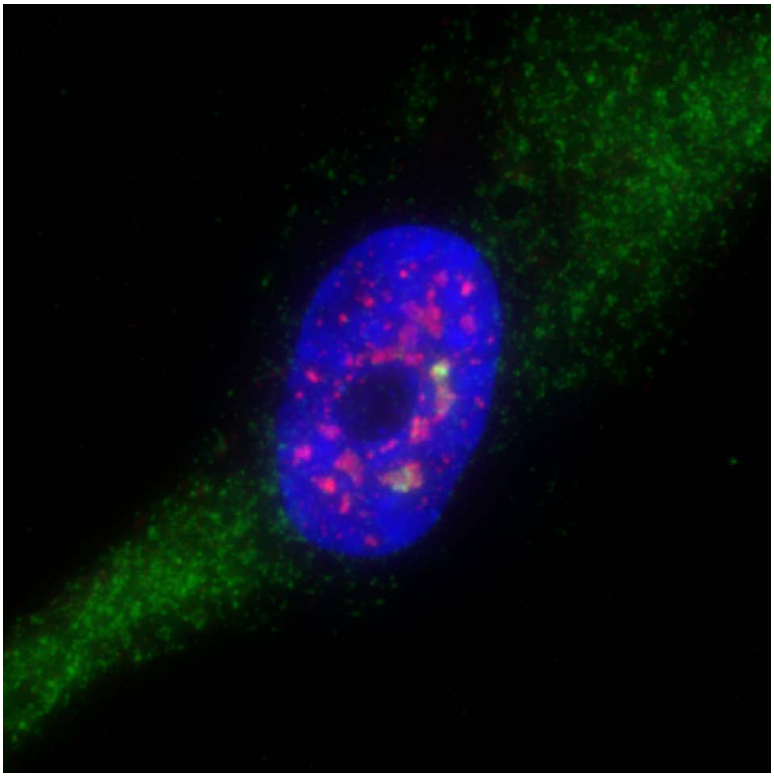

Figure 3. RNA from a specific gene interacts with sub-nuclear structures marked by enrichment for a specific protein

Collagen 1A1 RNA transcripts (green) in nuclei concentrate within sub-nuclear structures rich in RNA splicing factors, termed SC35 domains or “speckles”. The latter are labeled with an antibody to the spliceosome assembly factor SC35 (red). Photo by Kelly Smith.

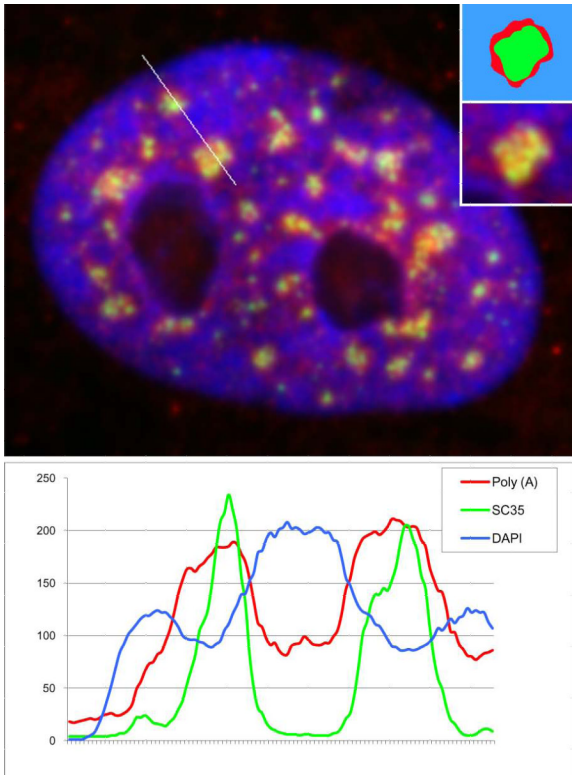

Figure 4. Quantitative comparison of signal distributions for endogenous poly A RNA and specific protein signals within a single nucleus

Poly-dT oligos can detect poly-A containing RNA (red) enriched in SC35 domains (green). Digital morphometrics using any of a variety of imaging software programs can quantify digital pixel intensities across an image, to map and compare precise boundaries of signal areas and intensities (Hall et al., 2006. Originally published in Anat Rec A Discov Mol Cell Evol Biol 228(7):664-75)).

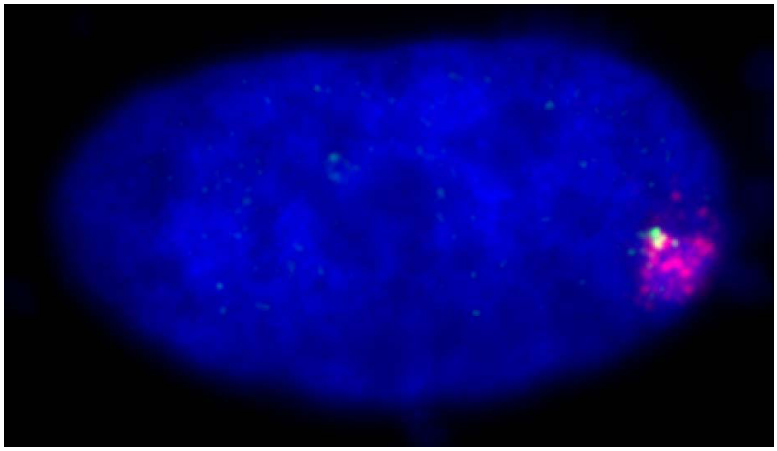

Figure 5. Identification and spatial arrangement of spliced versus unspliced RNA populations

A 2.7 kb probe to intron 1 of *XIST* labels an unspliced population of *XIST* (italics for gene symbols) pre-mRNA (green) associated with the site of transcription. In contrast a cDNA probe labels a much larger accumulation of *XIST* RNA (red) that lacks any intron sequence, providing evidence it is mature RNA, which stably binds the inactive X-chromosome in a female nucleus.

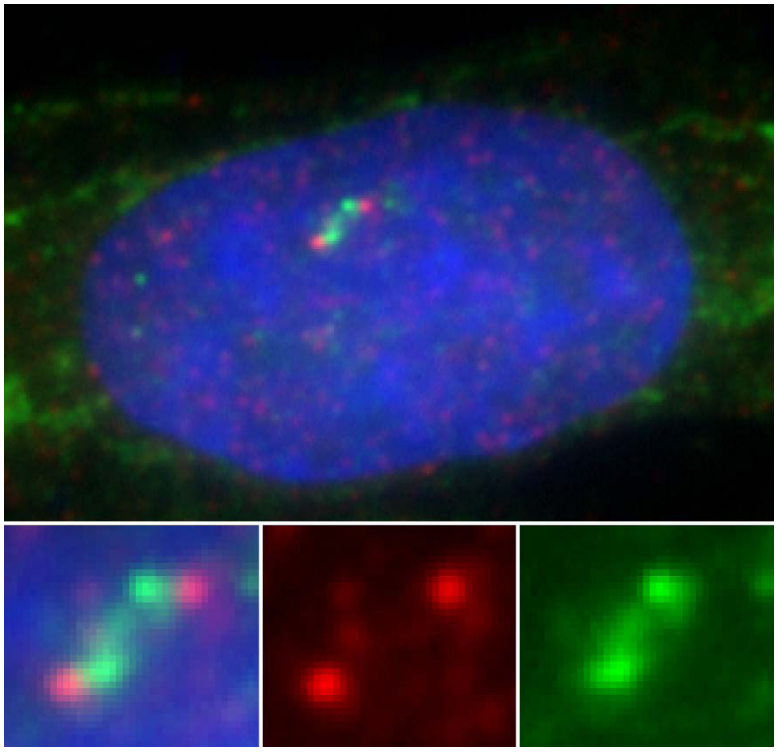

Figure 6. Simultaneous two-color detection of a gene and its cognate nuclear RNA shows precise displacement of RNA foci accumulating adjacent to the gene

Normal distribution of *DMPK* (dystrophia myotonica protein kinase) (red) and its RNA transcripts (green) detected with the same probe labeled in two different colors, using a sequential RNA and DNA hybridization approach (Smith et al., 2007. Originally published in J Cell Biol 178(6):951-64). Small inserts enlarge gene and RNA signals and separate color channels.

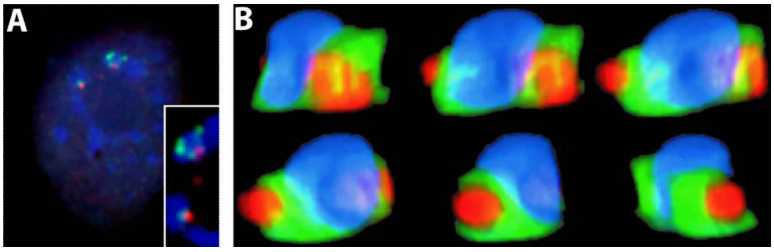

Figure 7. The relationship of a gene, its RNA, and a proteinaceous nuclear body in three colors and three dimensions

A) Here the same DNA probe labeled with two different moieties (biotin and digoxigenin) lights up *DMPK* (dystrophia myotonica protein kinase) (red) and its transcript (green) associated with an SC35 domain (detected with anti-SC35 antibody in blue), or B) using a different probe, *U2* (green) and its RNA transcript (red), associated with Cajal bodies (detected with coilin antibody in blue), are imaged by 3D rendering of deconvolution of Z-series optical sections (Smith et al., 2000. Originally published in Mol Biol Cell 11(9): 2987-98).

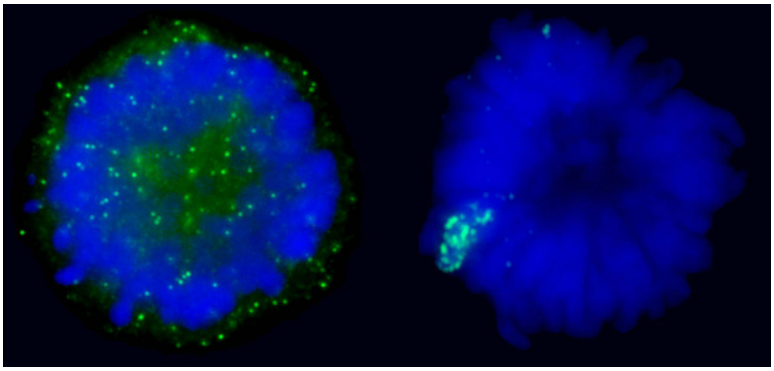

Figure 8. RNA detection in a metaphase cell reveals states of chromatin-bound or unbound *XIST* RNA

XIST RNA (green) is stably bound to chromatin during interphase (left cell), but is normally released at mitosis to the cytoplasm (bottom cell) (?) (Right cell). Manipulation of the phosphorylation state of chromatin controlled by Aurora B Kinase results in retention of *XIST* RNA binding to the chromosome in metaphase (Hall et al., 2009. Originally published in J Cell Biol 186(4):491-507).

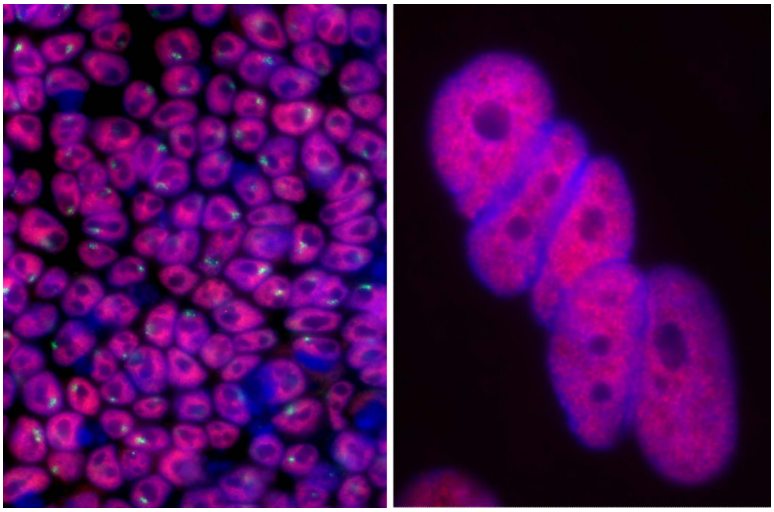

Figure 9. A novel assay with Cot-1 (repeat) DNA as a probe broadly detects nucleoplasmic hnRNA and discriminates regions of more or less active chromatin

Left) hnRNA (red) and *XIST* RNA (green) in human embryonic stem cells exhibiting precocious X-inactivation (Hall et al., 2008. Originally published in J Cell Physiol 216(2): 445-52). Right) Blue rims of DAPI stained DNA define the heterochromatic periphery of nuclei in differentiated myotubes, marked by sharply reduced hnRNA (red) expression (Hall et al., 2002; Tam et al., 2004. Picture originally published in J Cell Biol 167(2):269-79).

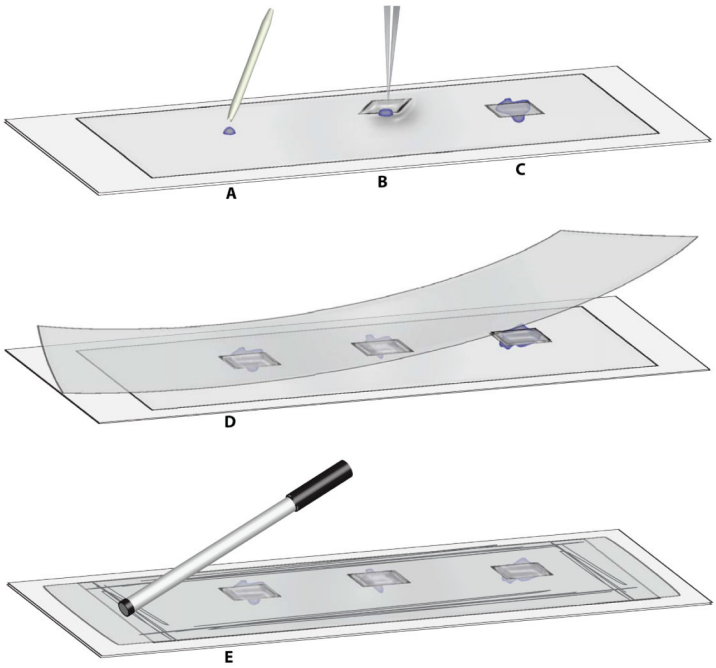

Figure 10. Building a "mini incubation chamber"

To protect delicate hybridization reactions during incubation, attach a sheet of parafilm to a glass plate and deposit the hybridization/detection solution onto the parafilm (A). Using forceps gently lower the coverslip (cell side down) onto the liquid (B-C). Place another sheet of parafilm over the coverslips (D), and seal the edges to prevent drying (E).