Chapter 6

Applications of RNA FISH for visualizing gene expression and nuclear architecture

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1 Introduction

Fluorescence *in situ* hybridization is not a singular technique, but a versatile experimental approach that provides ever more precise molecular information directly in the context of cellular structure. This chapter will focus on one major aspect of the molecular cytological approach: the detection of nuclear RNAs, together with their genes or other biochemical components of the cell. Improvements in the development and application of FISH in recent years have demonstrated the value of this approach for studying gene expression as well as processing, transport, and function of various cellular and viral RNAs. Although the basic technique described here is also applicable to detection of cytoplasmic mRNA, our focus will be on methods optimized primarily for high resolution, high sensitivity detection of nuclear transcripts, in precise relation to other nuclear components.

Standard molecular biology methods typically involve analysis of nucleic acids extracted from cells; therefore, they are limited by design to an averaged result obtained over a population of cells, often not physiologically homogeneous. Perhaps the most common use of RNA *in situ* hybridization is to assess gene expression on a single cell basis. If the goal is simply to assess which cells in a population are expressing a given RNA, then any *in situ* hybridization method which can detect that RNA within individual cells can be used, including autoradiographic or colorimetric methods. However, the detection of nuclear RNA by FISH can in some cases be advantageous for assessing expression of a gene within individual cells. As illustrated in *Figures 1* and *2* (see also *Plates 8* and *9*), the expression of myosin heavy chain RNA and collagen type I α1 (COL1A1) RNA are readily detected in the cytoplasm and nucleus of muscle fibres and human fibroblasts, respectively. However, the dystrophin RNA (*Figure 1*; see also *Plate 8*,
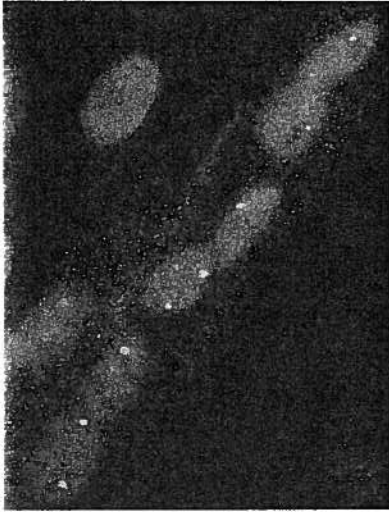
Figure 1 (See *Plate 8*) Detection of high and low abundance transcripts in nuclei versus cytoplasm of skeletal muscle fibres. Male human multinucleated myotubes were hybridized to detect muscle-specific myosin heavy chain (MyHC) and dystrophin RNAs. Most nuclei have two MyHC RNA foci (green) and a single dystrophin (on the X chromosome) RNA signal (red). While MyHC mRNA is evident in the myotube cytoplasm (green), the extremely low levels of dystrophin RNA in the cytoplasm are difficult to detect by FISH. Dystrophin gene expression is easily detected due to the large focal accumulation of nuclear RNA at the gene. A single undifferentiated myoblast nucleus (upper left) express neither MyHC nor dystrophin RNA (1).
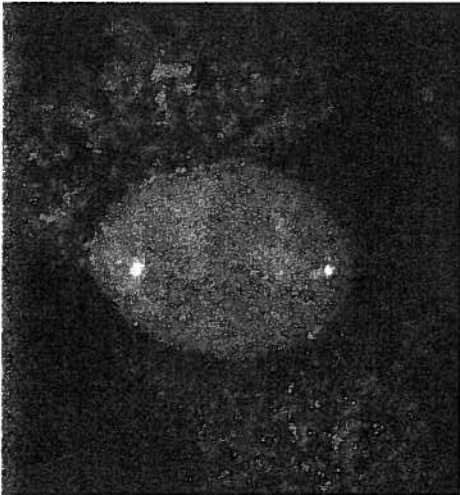

Figure 2 (See *Plate 9*) Visualization of mRNA distribution from gene to cytoplasm. Although procedures here are optimized for nuclear detection, in many cases nuclear and cytoplasmic transcripts can be detected simultaneously. In the human fibroblast shown here, sequential hybridizations to collagen type I α1 (COL1A1) RNA (red) and then the COL1A1 gene (green) reveals nuclear RNA foci emanating from the gene (overlap appears yellow), and distributed in the cytoplasm. More dispersed nuclear signal likely represents transcripts moving towards the nuclear envelope (19).

red), which is present at very low levels in the cytoplasm, is most easily detected at its site of synthesis in the nucleus, even using a probe that detects less than 1% of the full-length primary transcript (1). Although not widely appreciated, the most sensitive detection for some mRNAs will be derived from fluorescence hybridization to the nuclear RNA, since this can be the site of highest concentration in the cell, providing a discrete signal clearly distinguishable from background.

Although methods presented here will delineate which cells in a population express a given transcript, they can also be used for more powerful applications that put far greater demands on the experimental approach. For example, we may want to know how many or which alleles *within the same* nucleus are being expressed (*Figure 3*; see also *Plate 10*), or compare the processing or transport of transcripts from a normal and mutant allele within an individual heterogeneous cell (*Figure 7C* and *D*; see also *Plate 13C* and *D*). Such experiments require an approach that can deliver the best combination of extremely high sensitivity, resolution, and specificity, while being flexible enough to simultaneously label multiple DNA/RNA–protein components. Hence, the techniques summarized here will be of greatest value for studies in which the *intracellular* localization of RNA, not just its presence within the cell, is important for the biological or clinical questions investigated.

In this chapter, recent innovations and improvements to our standard laboratory procedures, and examples as applied to individual genes and transcripts are presented. The specific examples illustrated here derive from our recent work using RNA–DNA FISH in combination with immunocytochemistry to study both X chromosome inactivation and the relationship of gene expression and RNA metabolism to nuclear structure. In addition to basic science questions such as these, the ability to detect and localize small quantities of specific nucleic acids in single cells clearly has relevance for a variety of clinical applications. For a broader treatment of the development and applications of DNA/RNA *in situ*
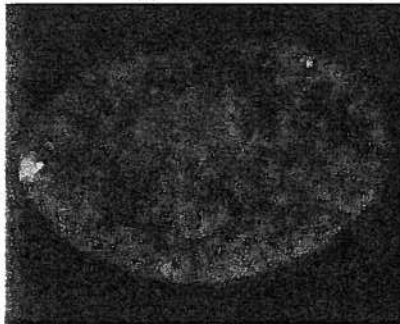
Figure 3 (See *Plate 10*) Nuclear RNA FISH can determine which alleles within a single cell are active. The UBE-1 gene is one that escapes inactivation on the inactive X chromosome. In contrast, the human XIST gene is expressed solely from the inactive X chromosome, and produces a large RNA accumulation (red) that 'paints' the inactive X. This contrasts with the much smaller UBE-1 nuclear RNA foci (green), more typical of signal for protein coding genes (7).

hybridization methodology, the reader is referred to previous reviews on this subject (2–4).

2 Cell preparation

The choice of a cell preparation protocol is determined primarily by the type of cell sample, the target RNA, and the objective of the experiment. To detect sequences within the nucleus, the protocol must permeabilize the nucleus sufficiently to allow the probe access to its target and, at the same time, fix the cell sufficiently to preserve labile RNAs and the integrity of structure. Many monolayer cells have extensive cytoplasm that requires extraction in detergent prior to fixation in order to adequately permeabilize the cell nucleus and allow detection of nuclear targets, hence the protocol detailed below uses this procedure. The detergent extraction method does not appear to significantly diminish or disrupt nuclear RNA, based on comparisons of RNAs that can be detected with or without the extraction (5–7). Because RNA is most vulnerable to degradation when cells are permeabilized prior to fixation, it is desirable to minimize the duration of pre-fixation steps. Often cytoplasmic transcripts can also be detected after 1–5 min of Triton treatment before fixation (*Figure 2*; see also *Plate 9*). However, these longer Triton extractions risk the loss of cytoplasmic mRNA and, if detection of cytoplasmic mRNA is the main goal, procedures using either very short (20–30 sec) Triton treatment before fixation, or longer Triton treatment *after* fixation, are preferable. We have also had success with the cell preparation protocol developed in Thomas Cremer's lab for DNA hybridization, which involves Triton and saponin extraction after fixation (8) (see also Chapter 7). Upon RNA hybridization, this protocol yields results that are very similar to cells extracted before fixation. The consistency of the results between the two quite different protocols supports the conclusion that extracting before fixation does not perturb the distribution of RNA in the nucleus.

It is important to note that not all cell types require permeabilization with detergent. We have found several cell types grown in suspension, which tend to have thinner cytoplasm, and require no extraction to detect nuclear sequences (e.g. lymphoma cells or human lymphocytes) (9, 10). These cells can simply be gently placed on to a multiwell slide (Cel-line) at the proper cell density and allowed to air dry briefly prior to fixation with formaldehyde (see below). Suspension cells can also be cytospun on to glass slides (11). This procedure flattens rounded cells, which is advantageous for detecting weaker signals, but less ideal for preserving cell morphology. Detergent extraction is also generally unnecessary in tissue sections, since nuclei at the surface of the tissue are often exposed during cutting of the section.

2.1 Detergent-extracted cell preparation

The standard protocol below is optimized for detecting nuclear RNA in monolayer cells (such as fibroblasts, epithelial cells, or skeletal muscle) and also works well for detecting DNA and most nuclear proteins.

Protocol 1

Extraction of monolayer cells

Equipment and reagents

- No. 1.5 coverslips
- Coplin jar
- Hanks' balanced salt solution (Invitrogen)
- 0.5% Triton X-100 in CSK buffer
- 200 mM vanadyl ribonucleoside complex (VRC, Invitrogen)

- Cytoskeleton (CSK) buffer:[a] 100 mM NaCl, 300 mM sucrose, 10 mM piperazine-N,N'-bis(2-ethane sulfonic acid) (Pipes) pH 6.8, 3 mM MgCl$_2$, 1.2 mM phenylmethylsulfonyl fluoride
- 4% paraformaldehyde in PBS (Ted Pella)
- 70% ethanol

Method

1 One to three days prior to fixation, grow cells to subconfluency on the coverslips.

2 Transfer coverslips to a Coplin jar and rinse several times with Hanks' balanced salt solution to remove media.

3 Rinse coverslips with CSK buffer at room temperature.

4 Gently extract cells with 0.5% Triton X-100 in CSK buffer for 1–5 min on ice. More structured cells, such as skeletal muscle fibres, require longer (e.g. 5 min) extraction. To prevent degradation of cellular RNA, include VRC in the extraction buffer at a final concentration of 10 mM. This may be crucial for detecting low copy transcripts.

5 Immediately fix cells for 10 min in 4% formaldehyde in PBS.

6 Rinse in 70% ethanol.

7 Cells can be stored in 70% ethanol at 4 °C for several weeks. Prolonged storage may cause RNA degradation. Ethanol can interfere with immunodetection of some proteins, which must be determined empirically. In such cases, cells can be stored for a few days in PBS.

[a] From methods in ref. 12.

2.2 Cytogenetic preparations

In some cases, it is desirable to examine RNA in a cytogenetic preparation where mitotic chromosomes can be evaluated with interphase nuclei. Cytogenetic preparations use methanol/ acetic acid fixation, which often does not preserve RNA well (13). In this case, RNA retention depends on properties of the individual transcript. For example, Epstein–Barr virus transcripts and XIST RNA are stable enough to be retained following a cytogenetic cell preparation, but transcripts from most cellular genes are not (7, 9).

A standard *in situ* harvest technique for cytogenetic cell preparations (14) has been adapted by K. Wydner, M. Byron, and C. Clemson. Using cells that have a high mitotic index bypasses the need to arrest cells in metaphase with colcemid. BrdU incorporation is included to elongate chromosomes and enhance banding.

Other cell preparation techniques that are aimed at more specialized structures including nuclear matrix (6) and nuclear halo preparations (15) are described in detail elsewhere (see also ref. 16).

Protocol 2

Preparation of cytogenetic cells and chromosomes

Equipment and reagents

- Tissue culture centrifuge
- Glass slides
- Steaming water-bath
- Slide warmer
- 100 mM BrdU (1000 × stock)
- Trypsin/EDTA: 0.05% trypsin, 0.53 mM EDTA (Invitrogen)
- Hanks' balanced salt solution
- Hypotonic solution: 75 mM KCl
- Fixative: 1:3 ratio of acetic acid/methanol

Method

1 4 h prior to harvest, add BrdU to the culture medium to a final concentration of 0.1 mM.

2 In the case of monolayer cells, trypsinize and wash cells by centrifuging at 150 g for 5 min and resuspending the pellet with 5 ml Hanks' buffer. Repeat the wash.

3 Resuspend cells in 1 ml of Hanks' buffer and gently add 7 ml of hypotonic solution. Incubate at 37 °C for 10 min.

4 Pellet by centrifugation at 150 g for 5 min.

5 Remove most of the hypotonic solution, leaving 1 ml with cell pellet. Completely resuspend the cells in this.

6 Add 1 ml of the fixative drop by drop. Continue to add more fixative to a final volume of 6 ml.

7 Pellet by centrifugation at 150 g for 5 min.

8 Repeat steps 6 and 7 four more times.

9 Resuspend cells to desired density in fixative and drop onto cleaned glass slides from a high point above slides, preferably in a humid environment for optimal spreading of chromosomes.

10 Pass each slide over a steaming water-bath and air dry on a slide warmer.

3 Probe preparation

The choice of DNA sequence used to make a probe can greatly affect the outcome of hybridization *in situ*. The size of the target sequence and the choice of whether a cDNA or a genomic DNA sequence is used will influence the intensity of the hybridization signal. Use of a cDNA probe is advantageous for detection of cytoplasmic mRNAs, however, the choice is more complicated for nuclear RNAs. The larger genomic sequence, with its many introns and flanking sequences, is more efficient for DNA hybridization or for detection of unspliced primary transcripts. We can detect gene signals with probes targeting as little as 1-2 kb in interphase nuclei and metaphase chromosomes, and specific nuclear RNA signals have been detected with oligonucleotides as small as 22 bp (20). Ideally, probes targeting 5-10 kb or more work best for single copy gene detection. The presence of repetitive elements in the probe is not a problem, provided Cot-1 DNA is added to compete these sequences (below). Likewise, with cloned plasmid DNA, it is not necessary to remove the inserted sequence of interest from the rest of the vector sequences.

Most commonly our probes are labelled by incorporating biotin or digoxigenin nucleotide analogues on to double-stranded DNA through a nick translation reaction. Detection of digoxigenin probes is more sensitive than that of biotin probes, but also generally produces more background. Endogenous biotin in some cell types can cause a significant problem with use of biotin labelled probes, but this difficulty is usually circumvented by the detergent extraction of the cell (above).

At the relatively high probe concentrations necessary for single gene detection (17), non-specific sticking of the probe can become more pronounced (discussed in ref. 16). Longer probe fragments tend to self-associate and form aggregates during the hybridization reaction, which can cause prohibitive background. For hybridization *in situ*, generating small probe fragments of less than 200 nucleotides in length is key for avoiding high background and optimizes penetration of cell structure.

Fragment size can be adjusted by varying the concentration of enzymes (DNase I and DNA polymerase) in the nick translation reaction. We have found it convenient to use a commercially available mixture of nick translation enzymes that are already calibrated and quality controlled for this purpose. The protocol detailed below uses the enzyme mix from Invitrogen BioNick Labelling Kit. The buffer/nucleotide mix from this kit is not used because it contains a mixture of biotin–dATP and 'cold' dATP, whereas we have found that inclusion of 100% labelled nucleotide results in the strongest probe signals. In addition, the efficiency of the reaction depends on the purity of the template DNA; CsCl preparations work consistently well in our hands.

Protocol 3

Nick translation of DNA probes

Equipment and reagents

- 20 °C water-bath
- Cesium chloride grade DNA
- 10 × nick translation buffer (NTB): 0.5 M Tris pH 7.5, 0.1 M $MgSO_4$, 0.5 mg/ml RNase-free BSA (Roche Diagnostics), 1 mM dithiothreitol
- 1 mM labelled dUTP (either biotin-16-dUTP or digoxigenin-11-dUTP) (Roche Diagnostics)
- Nucleotide mix of 600 µM each of dATP, dCTP, and dGTP (Roche Diagnostics)
- Enzyme mix from Invitrogen BioNick DNA Labelling System
- 5% sodium dodecyl sulfate (SDS)
- 0.5 M EDTA
- Salmon sperm DNA (Sigma)
- Ethanol

Method

1 For nick translating 0.5 µg of DNA, which serves for ten hybridizations, mix the following reagents in a microcentrifuge tube:
   - 2.5 µl of 10 × NTB
   - 2.5 µl nucleotide mix
   - 3.0 µl of 1 mM labelled dUTP
   - 0.5 µg DNA to be labelled
   - H_2O for a final reaction volume of 25 µl
   - 2.5 µl enzyme mixture

2 Incubate the reaction for 2.5 h at approx. 20 °C.

3 Add 2.5 µl of 5% SDS and 2.5 µl of 0.5 M EDTA.

4 Heat inactivate the reaction by incubating for 10 min at 65 °C.

5 Add 5 µg salmon sperm DNA.

6 Ethanol precipitate.

7 Wash pellet with 70% ethanol. (Omitting this step will result in probe with high background.)

8 Resuspend in 100 µl H_2O for a final concentration of ~5 ng/µl probe.

9 Probe can be stored at -20 °C for at least a year.

4 Hybridization to RNA

In addition to the quality of the cell preservation and probe preparation, there are several elements that are also important for successful hybridization to RNA. The quality of reagents used for hybridization (for example the brand of formamide) should be of high molecular biology grade and free of any RNase. Our laboratory routinely hybridizes cells overnight for convenience. However,

because the probe concentration recommended is generally in excess, hybridization times as short as 3 h are successful and may be advantageous to minimize cell/RNA degradation. Furthermore, the addition of VRC (see *Protocol 1*) to the hybridization mixture inhibits RNase activity.

In order to reduce cross-hybridization to highly repetitive sequences, excess unlabelled Cot-1 DNA is generally added to the probe mixture prior to hybridization, as are other non-specific nucleic acid competitors. These have proven to be critical for limiting sequence detection to specific RNA sequences. Cot-1 DNA repetitive sequences are species specific, thus the Cot-1 DNA used for hybridization should be derived from the same species as the target cells.

Hybridized probes can be indirectly detected with commercially available avidin or anti-digoxigenin antibodies that have been conjugated to Texas Red, rhodamine, or FITC (Roche). Alexa dyes conjugated to secondary antibodies or avidin (Molecular Probes) are another source for secondary detection and may have the advantages of being more sensitive, stable, and resistant to photobleaching. Each lot of secondary detector can vary in terms of titre and label content. Therefore, a range of concentrations should be tested initially to determine optimal conditions.

4.1 Basic RNA hybridization procedure

Protocol 4

Basic RNA hybridization procedure

Equipment and reagents

- Speed Vac lyophilizer
- Heat block at 80 °C
- 37 °C incubator
- Sheets of Parafilm
- Ethanol series: 70%, 95%, and 100%
- Nick translated probe (see *Protocol 3*)
- Competitor nucleic acid sequences: 10 μg each of sonicated salmon sperm DNA, *E. coli* tRNA, and Cot-1 DNA
- Formamide (ACS reagent grade, Sigma)
- Hybridization buffer: 0.4% BSA, 4 × SSC, 20% dextran sulfate, 40 mM VRC

- 20 × SSC stock: 3 M NaCl, 0.3 M sodium citrate pH 7.4
- Fluorochrome-conjugated secondary detection (either avidin or anti-digoxigenin antibody)
- RNase-free BSA (Roche Diagnostics)
- 10% Triton X-100 (Roche Diagnostics)
- 200 mM VRC (Invitrogen)
- Mounting media:[a] 0.1% phenylenediamine, 90% glycerol, PBS adjusted to pH 9.0 with sodium bicarbonate

Method

1 Dehydrate the cells on a coverslip through an ethanol series, air dry, and reserve for step 5, or remove from ethanol storage and rehydrate in 1 × PBS.

2 Lyophilize 50 ng of nick translated probe with 10 μg of each competitor sequence.

101

Protocol 4 continued

3 Resuspend the probe in 10 μl of 100% formamide (Sigma) and heat denature at 80 °C for 10 min.

4 Add 10 μl of hybridization buffer to the heat denatured probe.

5 Immediately spot probe mix onto a sheet of Parafilm, overlay with the coverslip (cell side down), and seal with a second sheet of Parafilm to prevent evaporation. Incubate at 37 °C in a humidified chamber for 3 h to overnight.

6 Rinse the coverslip in the following sequence for at least 15 min at each step:
   (a) 50% formamide, 2 × SSC at 37 °C.
   (b) 2 × SSC at 37 °C.
   (c) 1 × SSC at 25 °C.

7 Equilibrate in 4 × SSC for 1–2 min.

8 Detect the signal by incubating in either fluorochrome-conjugated biotin or fluorochrome-conjugated anti-digoxigenin antibody at a concentration of 3 μg/ml in 4 × SSC, 1% BSA, 40 mM VRC for 20 min–1 h at 37 °C.

9 Rinse for 10 min through each step:
   (a) 4 × SSC.
   (b) 4 × SSC, 0.1% Triton X-100.
   (c) 4 × SSC.

10 At this point, the signal may either be fixed for 10 min in 4% paraformaldehyde, PBS in preparation for subsequent procedures (see *Protocol 7*), or else it may be mounted and sealed for viewing. Cellular DNA can be counterstained with 4′,6-diamidino-2-phenylindole (DAPI) prior to mounting. To mount, add a drop of mounting media and seal the slide with nail polish.

ᵃ Commercial sources of pre-made mounting media are also available from Vysis and Vector Laboratories.

4.2 Oligonucleotide hybridization

Although targeting larger sequences where possible is preferable because their higher complexity reduces unwanted noise, especially when detecting DNA, oligonucleotide probes can be used for some RNA detection strategies. Oligos can be made or purchased (Operon, Biosource International) with a variety of labels (e.g. fluorescein, biotin) in varying numbers and positions. For some experiments, we have had success using 20–25 nt long oligos with a single biotin label at the 3′ end. The oligo should be HPLC purified for best results. These oligos have worked well for the detection of U2 and pre-U2 RNA in the nucleus (*Figure 4*) (20), as well as for detecting specific collagen introns (*Figure 11*; see also *Plate 16*) (21). However, differences in sequence content can greatly affect the hybridization and background properties of an oligonucleotide. While these probes can provide excellent specificity, their potential for spurious results or false hybridization should be well-controlled in the experimental design.
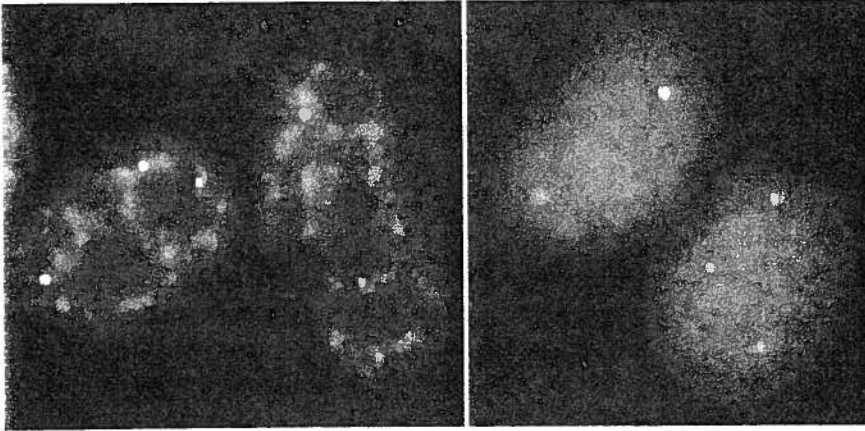
Figure 4 Oligonucleotide probes provide the high specificity needed to discriminate pre-U2 RNA from mature U2 RNA. The U2 gene produces a 199 nt long pre-U2 RNA, 11 nucleotides of which are removed to create the mature U2 snRNA. A 22 nt long 3' biotinylated oligonucleotide probe which is complimentary to nt 29–50 of U2 snRNA detects U2 in Cajal (coiled) bodies (bright foci) and splicing factor-rich speckles in HeLa cell nuclei (left panel). In contrast, an oligonucleotide of the same length complimentary to 15 nt of the mature U2 and 7 nt of the pre-U2 tail that are removed from the mature U2 detects only RNA in the Cajal body, under identical hybridization conditions (right panel). A negative control oligonucleotide with 15 nt of U2 sequence and 7 nt random sequence showed no specific signal (20).

Oligo hybridizations are performed using *Protocol 4* with minor modifications: a lower quantity of probe (5–20 ng) and a lower concentration of formamide (10–20%) in the hybridization buffer and in the wash. The formamide concentration must be adjusted for optimal efficiency depending on the length and GC content of the oligonucleotide probe. Competitor DNA is still included in the hybridization probe mix (10 μg each of ssDNA, tRNA, and Cot-1), and therefore the probe mix must be heat denatured before application to the slide. Another oligo hybridization protocol has been reported (18) and works equally well in our hands.

5 Hybridization to DNA

We have demonstrated several methods to target specifically and discriminate between RNA and DNA hybridization *in situ* (9, 16). The RNA hybridization procedure above precludes cross-hybridization to the gene, because essentially no hybridization to cellular DNA is detected if the double-stranded cellular DNA has not been denatured (9). In contrast, when the goal of the experiment is to strictly pinpoint the gene signal, residual RNA should be removed to prevent cross-hybridization.

If the gene of interest is transcriptionally active in cells studied, RNA can be removed by NaOH hydrolysis, RNase A pre-treatment, or RNase H post-hybridization (9, 19). NaOH treatment is the simplest and possibly most thorough method

and has the advantage that it simultaneously denatures DNA for subsequent hybridization. Hybridization to mitotic cells also provides a good control to verify DNA versus RNA signals, since these cells are not transcriptionally active, hence localized FISH signals represent DNA. To ensure hybridization is to RNA, either mitotic cells or a cell type that does not express the gene can be used as a negative control.

Treatment with RNase or NaOH does not necessarily remove RNA effectively or totally, although it is often assumed that it does. To control for the effective elimination of RNA when only DNA signal is desired, a good control is to omit denaturation of cellular DNA. If RNA has been effectively eliminated, this control should be completely negative.

5.1 Detecting heat denatured cellular DNA

Unlike detection of cellular RNA where preservation of the nucleic acid is key, detection of cellular DNA requires effective denaturation of double-stranded DNA. Our standard protocol uses heat denaturation, although when called for experimentally, we have used NaOH denaturation with variable success.

Protocol 5

Heat denaturation of DNA

Equipment and reagents

- Coplin jar
- Microwave oven
- 70 °C water-bath
- Thermometer
- 20 × SSC stock: see *Protocol 4*
- Formamide (ACS reagent, Sigma)
- Ice-cold ethanol series: 70%, 95%, 100%

Method

1 Equilibrate cells fixed on coverslips in 2 × SSC.

2 Microwave heat a solution of 70% formamide, 2 × SSC in a Coplin jar to 80 °C and transfer to a water-bath set at 70 °C. It is important not to allow the solution to sit at high temperatures for extended periods of time, as the pH of the solution may be altered and affect hybridization.

3 When the temperature is at exactly 74 °C, transfer the coverslip to the denaturation solution. Incubate for exactly 2 min. (The temperature should drop slightly but stay as close to 70 °C as possible.)

4 Transfer immediately to a Coplin jar containing ice-cold 70% ethanol and dehydrate through a 95% and 100% cold ethanol series.

5 Air dry.

6 Hybridize to denatured DNA according to *Protocol 4*. (VRC can be omitted from the hybridization buffer when preservation of RNA is not the objective.)

5.2 DNA FISH using NaOH denaturation and RNA hydrolysis

NaOH denaturation has worked well in our laboratory but has not proven as consistent as heat denaturation. Success at removing cellular RNA by this method also varies, depending both on the specific RNA target and the concentration of NaOH.

Protocol 6

Alkaline denaturation of DNA

Equipment and reagents

- Coplin jar
- Alkaline solution: 0.07 M to 0.2 M NaOH in 70% ethanol
- 70% ethanol
- 100% ethanol

Method

1 Incubate the coverslip in alkaline solution for 5 min.

2 Wash the coverslip for 5 min in 70% ethanol.

3 Repeat the wash in 100% ethanol.

4 Air dry.

5 Hybridize according to Protocol 4. (VRC may be omitted.)

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6 Multiple label techniques and applications

6.1 Coupling the detection of RNA with DNA

Experiments using multiple labels have innumerable applications for studying the spatial relationships between different cellular components. Distinguishing specific genes from their transcripts within the same nucleus has proven especially valuable for investigating the spatial organization of RNA metabolism. We have adapted our FISH protocols to visualize RNA in one colour and DNA in another. Using this technique, we have observed accumulations of transcripts from a few specific genes, for example COL1A1 and myosin heavy chain (MyHC), that extend in one direction away from the gene, suggesting vectorial transport of those RNAs away from their sites of transcription (Figure 5; see also Plate 11). This procedure will work with probes derived from the same sequence as long as they are differentially labelled. These probes are hybridized sequentially to differentiate between RNA and DNA as described in Protocol 7.
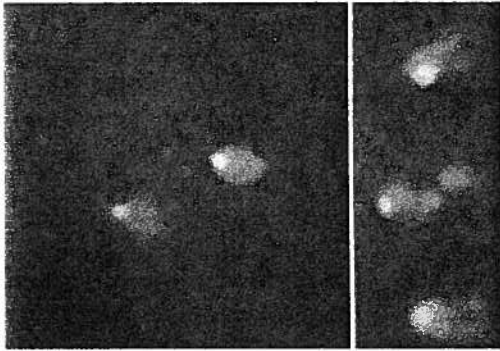
Figure 5 (See *Plate 11*) Simultaneous visualization of a gene and RNA emanating from that gene, shown for MyHC (right) and COL1A1 (left). Visualization of nuclear RNA (red) in direct spatial relation to the gene (green), can determine not only if both alleles are active, but also identify structural details with potentially important biological implications. Results of sequential RNA and DNA hybridizations (19) with a differentially labelled cDNA sequence were visualized using a dual bandpass filter (to avoid any optical shift). The two signals are not simply coincident, but rather the COL1A1I/MyHC RNA 'tracks' (red) are larger than the DNA signal and consistently positioned to one side of it (1, 19, 21). Often the prominent RNA signals are somewhat elongated and extend 0.5–3 μm beyond the gene (20–30 kb), which itself is at or below the resolution of fluorescence microscopy (0.2 μm). What may seem small differences in size and position by light microscopy are actually quite large and significant on a molecular scale.

Protocol 7

Sequential detection of RNA and DNA

Equipment and reagents

* See *Protocols* 4 and 6

Method

1 Hybridize first to RNA according to *Protocol* 4 using one of the forms of the labelled probe (e.g. digoxigenin) and detecting it in one colour (e.g. rhodamine).

2 Fix RNA signal by incubating in 4% paraformaldehyde, PBS for 10 min.

3 Wash twice, 5 min each, with PBS.

4 Follow *Protocol* 6 for alkaline denaturation of DNA and hybridize with a probe containing another type of label (e.g. biotin) and detecting the probe in a second colour (e.g. FITC).

5 If cellular protein will also be detected, fix DNA signal as in step 2, wash as in step 3, and proceed with *Protocol* 8. Otherwise, mount cells for viewing. ·

6.2 Coupling protein detection with FISH

Double- and triple-label experiments that combine RNA and/or DNA FISH with protein immunolocalization are invaluable for examining structural relationships between different genes or RNAs and other biochemical components of the cell. We have linked together hybridization and immunofluorescence techniques to determine whether specific genes or transcripts spatially associate with certain nuclear compartments or structures. In cases where they do, we have extended the technique to determine the precise alignment of gene, transcript, and nuclear body in triple-label experiments. Such triple-label experiments have shown, for example, that the accumulation of transcripts from the U2 snRNA locus does not necessarily mediate the preferential interaction between that gene and the Cajal body (Figure 10; see also Plate 15) (20).

Like the coupling of DNA and RNA FISH, linking the immunodetection of proteins with FISH occurs in sequential steps and can be affected by the order of the steps. We have found that some epitopes are destroyed by prior hybridization. In these cases, cells can be immunostained first and subsequently fixed to preserve that signal. If RNA detection is to follow, the addition of VRC to buffers during incubations with antisera helps prevent RNA degradation. Note also that some epitopes are compromised by exposure to ethanol, in which case cells can be stored in PBS instead but should be used within one to two days after fixation.

Triple-label experiments require the use of three distinct fluorophores. In addition to the more common fluorescein (FITC) and rhodamine or Texas Red agents (TRITC), we often use the coumarin derivative, aminomethylcoumarin (AMCA). AMCA-conjugated secondary antibodies can be used for labelling abundant protein antigens, but due to its low coefficient of extinction, AMCA is generally not intense enough to label transcripts or gene signals. In multiple label experiments, fluorochrome-conjugated secondary antibodies that have been cross-absorbed against multiple species are recommended to avoid cross-reactions.

Protocol 8

Immunostaining for proteins

Equipment and reagents

- Coplin jar
- Incubator at 37°C
- Primary antibody against protein of interest
- 1% BSA in PBS
- PBS
- Fluorescence-conjugated secondary antibody
- 0.1% Triton X-100 in PBS
- 4% formaldehyde in PBS

Method

1 Equilibrate coverslip in PBS.

Protocol 8 continued

2 Dilute primary antibody in PBS, 1% BSA and apply 20 μl to coverslip.

3 Incubate for at least 1 h at 37 °C.

4 Wash at 25 °C for 10 min each in:

   (a) PBS.

   (b) PBS, 0.1% Triton X-100.

   (c) PBS.

5 Dilute fluorescent-conjugated secondary antibody in PBS, 1% BSA to a concentration of approx. 3 μg/ml and apply 20 μl to coverslip.

6 Incubate for at least 20 min–1 h at 37 °C.

7 Repeat washes in step 4.

8 Fix signal in 4% paraformaldehyde, PBS for 10 min at 25 °C.[a]

9 Wash twice with PBS, 5 min each.[a]

10 Proceed with *in situ* hybridization protocol.[a]

[a] When the experiment calls for hybridization after immunodetection of specific proteins.

6.3 Chromosome paints and RNA FISH

Chromosome paints have proven useful for identifying individual chromosomes in cytogenetic preparations of metaphase cells and in delineating the boundaries of chromosome territories in interphase nuclei. An interesting example of this application is the study of the XIST RNA, which initiates inactivation of the X chromosome in mammalian nuclei (7). The chromosome paint when coupled with RNA hybridization to XIST delineates which X chromosome is inactivated and to what extent XIST RNA coats that chromosome (*Figure 6*; see also *Plate 12*). As with other sequential hybridizations, RNA species are detected first and then

Figure 6 (See *Plate 12*) Hybridization to RNA in nuclei was key for discovering the novel role of XIST RNA, which 'paints' its parent X chromosome and induces its inactivation. Comparison of human XIST RNA to X chromosomal DNA; the latter was detected using a library of X chromosome-specific DNA fragments. In the cell line depicted (karyotype 47, XXX), two of the X chromosomes (red) are inactivated and coated by XIST RNA (green), whereas the active X chromosome (lower left) is not (7).

the chromosomal DNA is hybridized (see above). In some cases in which a chromosome paint is weak or not fully delineating the chromosome, we have obtained a more complete hybridization using cells prepared according to Kurz et al. (8) (see also Chapter 7). Not all commercial sources of chromosome paints work equally well in our hands. We have had success with paints that are prepared by degenerate PCR amplification of FAC-sorted chromosomes, such as CamBio's STAR*FISH paints. The following is a protocol for DNA hybridization using a chromosome paint as a probe, which can be coupled to RNA detection as mentioned above.

Protocol 9

## Hybridization with chromosome paint

Equipment and reagents

- See *Protocol 5*
- 37°C water-bath
- 70°C heat block

- 20 μg Cot-1 DNA
- STAR*FISH paint (Cambio/Vysis)

Method

1 Heat denature the cellular DNA according to *Protocol 5*, steps 1–5.

2 Lyophilize 20 μg of Cot-1 and resuspend in 15 μl of commercially labelled STAR*FISH paint pre-warmed in a 37°C water-bath for 30 min.

3 Heat denature the probe at 70°C for 10 min.

4 Reanneal with the provided competitor DNA at 37°C for 20 min to minimize non-specific hybridization.

5 Apply to coverslip and continue with *Protocol 5*, step 6.

6.4 Differentiating transcripts with intron and cDNA probes

Visualizing RNA detected with a cDNA probe in one colour and a probe specific for intron sequences in another can reveal much about the splicing of a transcript. When combined with immunolocalization of splicing factors, this can demonstrate structural information about where and how this process occurs (19). For example, Johnson et al. (21) used this strategy to compare the fate of RNAs produced from the normal and the splice-defective allele of collagen Iα1 in heterozygous human cells from a patient with osteogenesis imperfecta (*Figure 7*; see also *Plate 13*). Such analyses can demonstrate whether the mutant transcript is degraded or retained within the nucleus, and provide a means to better understand where and why the splice-defective transcript may fail to be transported to the cytoplasm.
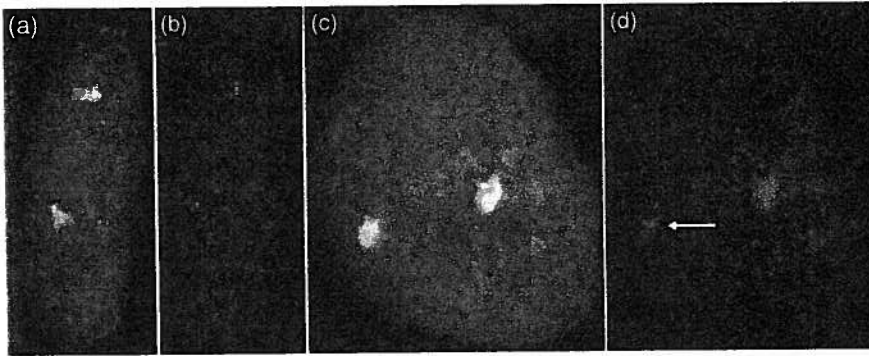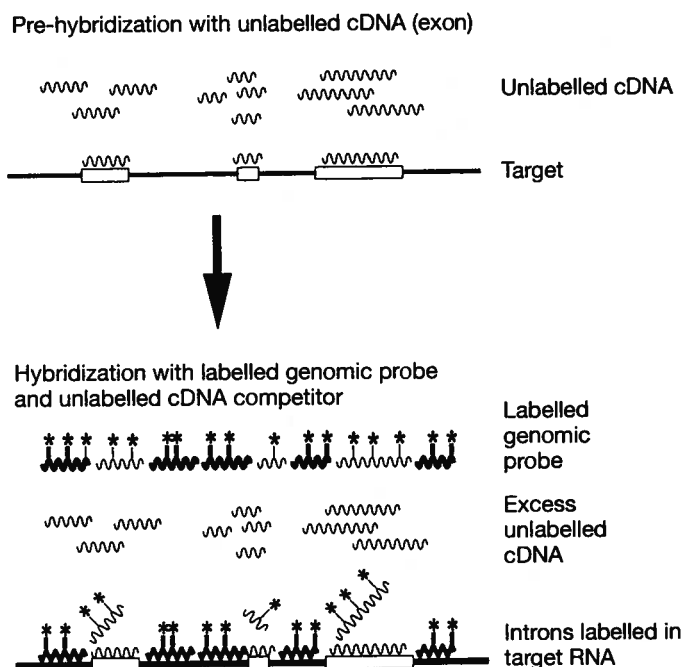
Figure 7 (See *Plate 13*) High resolution analyses of intron and exon RNA distributions can localize sites of splicing and detect splice defects from an individual allele in a heterozygous cell. Simultaneous RNA hybridizations with probes for COL1A1 intron 26 and cDNA in normal WI-38 fibroblasts (A, B) versus O54 mutant cells (C, D). The mutant cell line is heterozygous for a mutation in the splicing of intron 26. Both alleles in the normal cell show a limited overlap and polar orientation of intron 26 (red) with respect to the cDNA accumulation (green). In contrast, in the mutant cell line, transcripts from the mutant allele are readily discerned from the normal (arrow) by the more uniform distribution of intron 26 (red) throughout the cDNA track (green) (21).

We have successfully hybridized to introns using oligonucleotide probes (see above) and specific intron sequences amplified from genomic DNA by PCR (*Figure 11*; see also *Plate 16*). The intron of interest is amplified following the instructions provided in the PCR core kit (Roche) and then purified by phenol/chloroform extraction from a low melt agarose gel. The DNA is nick translated according to *Protocol 3* and used for hybridization as detailed in *Protocol 4*. To verify specificity, we typically hybridize the PCR probe simultaneously with a cDNA probe containing a different label (*Figure 7*; see also *Plate 13*).

6.5 Exon suppression hybridization: an example of the use of specific competition

In order to visualize the signal generated by essentially all introns in a transcript, we devised a strategy that blocks detection of exons by hybridizing with a genomic DNA probe in the presence of excess, unlabelled cDNA competitor. This 'exon suppression' strategy (21) makes it feasible to detect with a single probe the collective distribution of introns, and it illustrates a broader concept for how competition can enhance the capabilities of the FISH approach. The strategy is outlined in *Figure 8* and illustrated in *Figure 9* (see also *Plate 14*). We have used sequential hybridizations, first with a labelled COL1A1 cDNA followed by exon suppression hybridization, to dissect the COL1A1 RNA transcript track (21). This analysis has shown that the bulk of the 51 introns in transcripts from the COL1A1 gene are removed at one end of the transcript accumulation, at or near the gene.

Pre-hybridization with unlabelled cDNA (exon)

Unlabelled cDNA

Target

Hybridization with labelled genomic probe
and unlabelled cDNA competitor

Labelled
genomic
probe

Excess
unlabelled
cDNA

Introns labelled in
target RNA

Figure 8 Outline of the strategy for exon suppression hybridization. This technique was
designed to visualize the collective distribution of all intron transcripts from a highly complex
gene, by competing away any hybridization to exons by a labelled, full-length genomic DNA
probe. Hybridization to exons is suppressed by a preliminary hybridization step with unlabelled,
full-length cDNA, and addition of excess cold cDNA to the hybridization reaction (using a
labelled genomic probe). Only labelled probe fragments containing intron sequences hybridize
to their targets and are detected (solid bars).

This procedure is designed to localize the majority of the introns transcribed
in a complex gene such as collagen. We note that the exon suppression tech-
nique would not necessarily detect a deviation in the level or distribution of any
one intron, because a single intron may represent too small a proportion of a
large, complex primary transcript. Also, it is important to assess the efficiency of
suppression using a control experiment in which labelled, full-length cDNA is
competed by unlabelled, full-length cDNA.

Protocol 10

Exon suppression

Equipment and reagents

- See *Protocols 3* and *4*
- Full-length cDNA

- Full-length genomic DNA
- DNase I (Roche)

Protocol 10 continued

Method

1 Digest >10 μg of full-length cDNA with DNase I to yield fragments approximately the same size as a nick translated probe.

2 A pre-hybridization step with 2–4 μg of the unlabelled, digested cDNA (step 1) is necessary to saturate the exon binding sites. 10 μg of each competitor, tRNA, Cot-1, and ssDNA are included in this pre-hybridization solution. Hybridize from 2 h to overnight at 37°C and wash without detecting.

3 In the second step, hybridize overnight with 50 ng nick translated probe that has been generated from a full-length genomic DNA clone. Include an additional 4 μg of the unlabelled, DNase I digested cDNA (step 1) and 10 μg each of tRNA, Cot-1, and ssDNA in this hybridization.

4 Wash and detect as in *Protocol 4*.
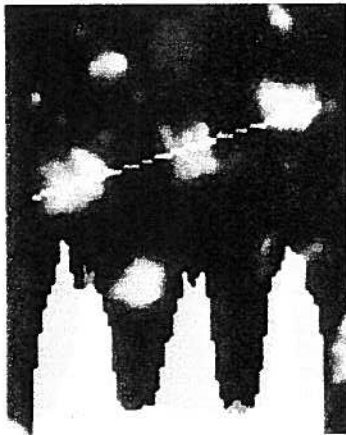
Figure 9 (See *Plate 14*) Use of selective competition procedures coupled with high resolution quantitative microscopy can enhance the power of FISH analyses. An 'exon suppression' strategy (diagrammed in *Figure 8*) was devised to determine the collective distribution of COL1A1 intron RNAs, encompassing 50 different introns (21). (Top) The collective intron RNA signal (red) was directly compared to the distribution of spliceosome assembly factor SC-35 (green), demonstrating that most if not all introns are removed in the periphery of the SC-35 domain. (Bottom) Microfluorimetry allows high resolution quantitation of the relative distributions of these two fluorescent signals. The computer reads out the signal intensity of each pixel along the blue line, showing quantitatively the removal of introns (splicing) occurs at the periphery of the SC-35 domain. Since RNA detected with a cDNA probe distribute throughout the domain (not shown), this indicates that spliced transcripts enter the domain after splicing (21).

7 Visualizing and analysing results

The hybridization protocols presented here can in principal be coupled with various kinds of detector reagents, for visualization by transmission microscopy

(colorimetric detection), electron microscopy (gold or electron dense labels), or epifluorescence microscopy, as we describe here. Fluorescence microscopy provides a powerful combination of sensitivity, resolution, and multicolour labelling. Where the goal is simply to determine which cells within a culture or tissue are expressing a product, colorimetric detectors (e.g. alkaline phosphatase), may be as good or better than fluorescence because they avoid common difficulties with autofluorescence in tissues. However, such procedures do not provide adequate resolution for most studies of intracellular structure. Electron microscopy provides greater resolution than fluorescence, but requires thinner specimens, more sample preparation steps (potentially destructive to RNA), and thus far has proven less sensitive for detection of nucleic acids *in situ*.

FISH has been demonstrated to have robust sensitivity and resolution for the detection and localization of single genes and specific nuclear RNAs. It can routinely detect single genes or DNA sequences of 2–4 kb or more with high efficiency. As illustrated in the examples above, relatively low-level transcripts, such as intron-containing pre-mRNAs or pre-U2 RNA, can be readily detected with probes targeting as little as 22 bp; however, this sensitivity is likely augmented by small but localized concentrations of these transcripts. When RNA is dispersed throughout the cell or nucleus, one may see tiny fluorescent spots that likely represent the detection of individual mRNAs (~2–4 kb). However, this does not mean that the sensitivity of detection is a single transcript per cell. While it may be possible to visualize fluorescence from an individual transcript, unless there are many transcripts dispersed through the cell it is unlikely that one could confidently distinguish this from background. Hence, RNA distribution within the cell will impact substantially on the sensitivity of detection, since more localized signals are more easily discerned from background, as illustrated in *Figure 1* (see also *Plate 8*).

7.1 Microscopy

Because the confocal microscope can eliminate a significant proportion of fluorescent signal and can rapidly photobleach samples, we prefer to use a wide-field microscope for most purposes, even for most 3D analysis. (Confocal microscopy may be necessary for 3D studies of larger, brighter signals in thick tissue specimens; see Chapter 7.) High magnification, high numerical aperture lenses also greatly facilitate the detection of signals arising from single copy genes and low abundance RNA, and a microscope equipped with a ×100, 1.4 NA oil immersion objective is recommended. The size of most gene signals is at or below the level of resolution provided by a ×100 objective (approx. 250 nm) and, therefore, sequences up to 50–100 kb (or more) in length will produce a signal that appears as a single, small point of light. Because the limits of resolution are large relative to the size of individual molecules, this means that any consistent spatial separation evident between two signals has biological significance on a molecular scale *which is often overlooked* (*Figure 10*; see also *Plate 15*).

Filter selection also plays a critical role in fluorescence microscopy, particularly when precise alignment of signals is the experimental objective (see

113
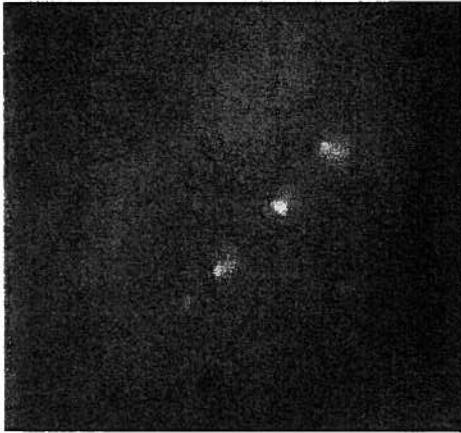
Figure 10 (See *Plate 15*) Triple-label detection of DNA, RNA, and protein. A HeLa cell nucleus stained with an anti-coilin antibody to visualize Cajal (coiled) bodies (blue), coupled with sequential RNA–DNA hybridization and two-colour detection of U2 RNA (red) and DNA (green), using a 6.1 kb genomic U2 locus probe. This nucleus shows a Cajal body with each of the three U2 gene foci, as well as a separate RNA signal (20).

Chapter 2). A triple bandpass filter set that employs a single beam splitter and emission filter incorporated into the body of the microscope (available from Chroma Technology) guarantees precise superimposition of signals from different excitation wavelengths. All microscope systems should be evaluated for optical shifts. A shift that is optical and not biological will result in one colour being consistently shifted in the same direction relative to the other.

Hundreds of cells can easily be viewed directly through the microscope by independent examiners, and in multiple experiments, which avoids biases that can be introduced by more selective analysis of a few imaged cells. Precise structural analysis can generate details concerning both the relative positions and morphology of two or more nuclear constituents which can lead to significant insights into nuclear processes (*Figures 2* and *5*; see also *Plates 9* and *11*).

7.2 Digital imaging

In recent years, the recording and analysis of microscopy data has been greatly aided by the development of CCD cameras specifically adapted for use with the light microscope. Generating digital images with CCD cameras offers several advantages over conventional photography, including the ease and speed of acquisition and storage, flexible manipulation of image size and contrast, signal morphometry and quantification, and the ability to capture and render 3D data sets.

While digital imaging can speed and enhance data analysis, care must be taken to record data in a manner that accurately reflects the signals in the actual sample. For example, because CCD cameras offer high sensitivity and digital images are easily manipulated, artefactual data can be generated by signals

bright enough to 'bleed-through' the emission filter (e.g. bright TRITC signals through the FITC emission filter), background in the sample, or simply electronic noise, which can be misinterpreted as real signal. Conversely, very weak signal can be integrated over long exposures enabling visualization of signal not seen through the microscope. Digital imaging is an especially powerful technique because it can be used to quantify morphometric and intensity data. A 12-bit CCD camera has greater dynamic range than the human eye and, signal properties can be readily quantitated. However, results can be greatly affected by the way the measurement threshold is set. Microfluorimetric data is most meaningful when determined relative to a standard signal within the same image. As illustrated in a recent study where it was shown that a significantly increased accumulation of COL1A1 RNA was present at the mutant versus normal COL1A1 allele in osteogenesis imperfecta (*Figure 7*; see also *Plate 13*). Microfluorimetry can also be used to indicate the relative distributions of two different labels in the same nucleus, as shown for the analysis of spliced and unspliced COL1A1 nuclear structure (21) transcripts within a splicing factor enriched nuclear compartment, the SC-35 domain (*Figure 9*; see also *Plate 14*).

Although substantial 3D information can be gleaned by simply adjusting the focus while looking through the microscope eyepiece, more extensive analysis is sometimes important. 3D images can be generated by collecting z-series stacks of images from a wide-field microscope and applying a photon reassignment algorithm to reconstruct the image in 3D (for details see *Figures 12* and *13*, and *Plates 17* and *18*). Alternatively, a confocal microscope can be used to acquire 3D information, as discussed further in Chapter 7.

Figure 11 (See *Plate 16*) The spatial distribution of two different introns reflects the temporal order of splicing of COL1A1 transcripts. Detection of COL1A1 RNA intron 24 (green) using overlapping biotin labelled oligonucleotides simultaneously with intron 26 (red) using a nick translated probe (labelled with digoxigenin) from a PCR-amplified fragment (21). Because intron 24 (green) is spliced later, it is retained throughout much of the globular RNA 'track', whereas intron 26 (red) is only present in a small focus at one end of the track (near the gene) (see ref. 21).

115
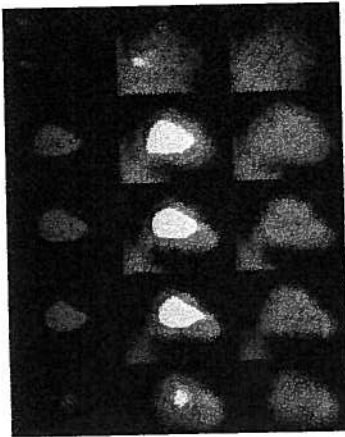
Figure 12 (See *Plate 17*) Valuable 3D information can be obtained simply by capturing images of distinct focal planes along the z-axis. In this example, a simplified approach to 3D analysis was used to make the point that collagen RNA (red) is found *within* an SC-35 domain (green), rather than just above or around it. By manual optical sectioning, the two signals, which both span several optical sections (~0.2 μm apart), can be seen going in- and out-of-focus in essentially the same focal planes, and clearly overlap (yellow) within the inner planes of the SC-35 domain (19). For higher resolution analysis, particularly for smaller structures, a more sophisticated 3D analysis would be more useful (*Figure 13*; see also *Plate 18*).
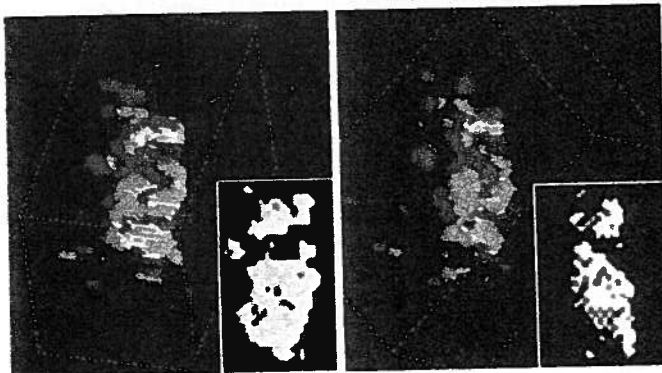
Figure 13 (See *Plate 18*) Deconvolution and 3D rendering shows that XIST RNA distributes throughout the interphase X-chromosome territory, not just above or below it. Here optical sections captured with a CCD camera were deconvolved to remove out-of-focus light, and data from all planes then rendered to display the object in three-dimensions. The two panels depict a 90° rotation in the projected three-dimensional reconstruction (7). Each panel shows a surface view of the 3D object from that angle, showing XIST RNA (red) and X chromosome DNA (green), with areas of overlap (white). The insets show a similar 3D object is seen when only pixels with overlap are viewed.

8 Concluding remarks

This chapter provides a methodological overview and detailed experimental protocols, for the study of gene expression and RNA metabolism, as it relates to

nuclear structure and genome/chromosome organization in the context of individual cells. Molecular cytology is invaluable for investigating not only cell type-specific gene expression during development, but also how complex molecular and biochemical processes of gene expression are actually integrated and working within the cell structure. For example, the *in situ* visualization of stable, spliced XIST RNA was essential for revealing the unprecedented 'painting' of the X chromosome by this non-coding RNA, key to its role in X inactivation. Other examples of the singular abilities of *in situ* analysis are the ability to visually discriminate differential transcription or splicing from alleles within a single nucleus, or address where a block to transport of mutant transcripts occurs within that nucleus. It is increasingly appreciated that the intracellular or intranuclear localization of genes, RNAs, and proteins *in situ* may be critically important for understanding their roles both in normal cells and disease.

With the continued development of new hybridization strategies, new types of probes, and innovative developments in microscopy for fixed and living cells, the power and versatility of this approach will be increasingly appreciated and essential for understanding fundamental aspects of cell function intimately intertwined with structure.

Acknowledgements

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