It is advisable to determine empirically the optimum annealing conditions for each primer.

A second problem is primers that do not label efficiently or do not prime efficiently due to the formation of competing structure within the primer or the target. These problems may require using alternative primers. If several primers on the same target do not produce satisfactory signals, the target may not be abundant enough. Enriching the RNA sample for the target or optimizing the KCl concentration in the reverse transcriptase reaction may help.

A third problem that is usually diagnosed during the evaluation of primer extensions is that of ribonuclease contamination of samples. This is most often seen when a strong stop is observed at a position (or several positions) equivalent to a dideoxyTTP-induced stop at an A residue in the target RNA. The A will almost always be preceded by a U in the sequence. This usually indicates that the target RNA has been hit by a pancreatic-type (RNase A) nuclease that prefers UpA sites for cleavage, producing a strong stop at the cleavage site.

Difficulty in generating a satisfactory sequencing ladder usually can be remedied by adjusting the concentration of the dideoxyNTP stocks used until an appropriate distribution of stops is obtained for each nucleotide.

Acknowledgments

We thank Uwe von Ahsen for help with aniline cleavage and Sandra Wolin, Carol Dieckmann, Dave Higgs, David Stern, and Dave Brow for sharing *in vivo* DMS experiences with us. This work was supported by NIH Grant GM40478.

[33] Sensitive and High-Resolution Detection of RNA *in Situ*

By PASCAL CHARTRAND, EDOUARD BERTRAND, ROBERT H. SINGER, and ROY M. LONG

Introduction

Unlike other molecular approaches, *in situ* hybridization offers the advantage of studying RNA in the complex environment of the eukaryotic cell. Also, due to their higher spatial resolution, fluorophore-labeled probes are increasingly replacing enzymatic and radioactive methods used previously to detect cellular RNA. For this reason, fluorescence *in situ* hybrid-

0076-6879/00 $30.00

ization has been applied successfully not only for the simple detection of mRNA,[1] rRNA,[2] tRNA,[3] snRNA,[4] and snoRNA,[5] but also to study the transcription,[6] processing,[7] export,[8] and localization[9,10] of these RNA in several types of eukaryotic cells.

This article provides protocols for fluorescence *in situ* hybridization (FISH) on RNA in yeast and tissue culture cells. These protocols use fluorescent-labeled probes, but are applicable to other detection techniques based on biotin–streptavidin or digoxigenin.

New microscopic designs and methods for image acquisition and analysis are particularly useful when employing fluorescence as a detection method. Algorithms that can remove out-of-focus light and quantitate fluorescent signal[6] have provided powerful means by which to extract information from acquired digitized images. An important consideration in analyzing these images is that decisions on scaling and background threshold settings must be made objectively.[11] While this is not the subject of this article, it will be ultimately the most important component of the data presentation.

Oligonucleotide probes have significant advantages over long probes and some disadvantages. Advantages are that they can be synthesized rapidly from the abundant and growing sequence information in databases. They can be targeted to specific genomic regions, free of repetitive sequences, or the isoform-specific regions of RNA, such as the 3′UTR.[12] Because large amounts of oligonucleotide probes can be made (milligram amounts, enough for thousands of experiments), the probes serve as a consistent reagent over a period of years. Conjugation of the probes with fluorochromes or other reactive groups can be controlled chemically and quality assured. Unlike probes prepared enzymatically, every probe is identical, allowing for better reproducibility in the experiments. Hybridization times are short because of the low complexity and signal-to-noise ratios are higher. A disadvantage is that they are small and hence give a fraction

[1] K. L. Taneja, L. M. Lifshitz, F. S. Fay, and R. H. Singer, *J. Cell Biol.* **119,** 1245 (1992).
[2] I. B. Lazdins, M. Delannoy, and B. Sollner-Webb, *Chromosoma* **105,** 481 (1997).
[3] E. Bertrand, F. Houser-Scott, A. Kendall, R. H. Singer, and D. R. Engelke, *Genes Dev.* **12,** 2463 (1998).
[4] A. G. Matera and D. C. Ward, *J. Cell Biol.* **121,** 715 (1993).
[5] D. A. Samarsky, M. J. Fournier, R. H. Singer, and E. Bertrand, *EMBO J.* **17,** 3747 (1998).
[6] A. Femino, F. S. Fay, K. Fogarty, and R. H. Singer, *Science* **280,** 485 (1998).
[7] G. Zhang, K. L. Taneja, R. H. Singer, and M. R. Green, *Nature* **372,** 809 (1994).
[8] D. A. Amberg, A. L. Goldstein, and C. N. Cole, *Genes Dev.* **6,** 1173 (1992).
[9] A. Ephrussi, L. K. Dickinson, and R. Lehmann, *Cell* **66,** 37 (1991).
[10] R. M. Long, R. H. Singer, X. Meng, I. Gonzalez, K. Nasmyth, and R-P. Jansen, *Science* **277,** 383 (1997).
[11] F. S. Fay, K. L. Taneja, S. Shenoy, L. Lifshitz, and R. H. Singer, *Exp. Cell Res.* **231,** 27 (1997).
[12] E. H. Kislauskis, Z. Li, R. H. Singer, and K. L. Taneja, *J. Cell Biol.* **123,** 165 (1993).

of the signal of a probe containing much more sequence. Although "cocktails" of oligonucleotide probes can be generated, this becomes expensive. Also, they are not applicable if the sequence information is not known. Generally, oligonucleotide probes are more difficult for detecting DNA sequences by FISH, as signal levels are low. As many as 10 probes (500 nucleotides total) will be necessary.[6] In this case, larger probes containing 10 kb of genomic sequence are preferable. Similarly, detection using cRNA probes is also a viable approach in all cases using enzymatically prepared probes. Large probe sizes (>200 nucleotides) have to be removed to avoid a higher background. The key to success is that the iteration of many small probes on the template leads to detection. The following protocols report on preparation and use of this variety of probes. Continuously updated information may be found at www.singerlab.org.

Preparation of Probes

Oligonucleotide Probes

DNA oligonucleotide probes are currently preferred for *in situ* hybridization. They are synthesized chemically, which allows the incorporation of amino-modified nucleotides [usually an aminoallyl(dT)] at specific positions in their sequence. Each free amine can then be coupled to a fluorophore after synthesis. The main advantage of direct labeling of probes resides in the high signal-to-noise ratio of these probes compared to indirect detection methods using antibodies. Even if the absolute signal is higher using fluorescent-labeled antibodies, they will contribute to a higher background. Also, these probes are small enough to penetrate easily in the cells (a 50 base oligonucleotide is 50 times smaller than the Fab fragment of an antibody) and can be washed away under mild conditions, compatible with the retention of intracellular RNA, and reduce the background fluorescence signal.

Design and Synthesis. Usually, between two and six probes of 50 nucleotides complementary to a given RNA are used to detect this specific RNA in a cell. These probes are designed to have five amino-modified T per oligonucleotide, each separated by a stretch of 10 nucleotides and, if possible, a total G + C content of 50% (if not possible, all the oligonucleotides should be designed to have the same melting temperature). The oligonucleotides can be synthesized on a DNA synthesizer on a low-scale 0.2 μmol column, which will give enough quantity of oligonucleotides for years. The amino-modified dT used should contain a six-carbon arm between the base and the free amine in order to increase the coupling of the fluorophore.

Purification. After synthesis and deprotection, the oligonucleotides are

purified on an acrylamide gel in order to remove the lower molecular size oligonucleotides.

1. Set up a 10% acrylamide/urea gel with large wells.
2. Resuspend the dry oligonucleotide in a 9:1 formamide: 1× TBE buffer (100 m*M* Tris, pH 8.3, 100 m*M* boric acid, 2 m*M* EDTA) with dyes. Load and run until the xylene cyanol dye reaches the bottom of the gel.
3. Visualize the DNA under UV. Cut the band, chop up into fine pieces, and place in a 50-ml tube with 25 ml of distilled water. Elute overnight at 37°.
4. Repeat the extraction twice with 15 ml of 10 m*M* NaCl for 2 hr at room temperature. Lyophilize each extraction and resuspend in 1 ml of distilled water.
5. Load the extract on a Sephadex G-50 column (Pharmacia, Piscataway, NJ) and collect the fractions. Take the OD_{260} of each fraction and pool those containing pure oligonucleotides.

The oligonucleotides can also be purified by reversed-phase chromatography on a Poly-Pak column (Glen Research, Herndon, VA).

Labeling of Oligonucleotide Probes with Terminal Transferase

Another approach used for the labeling of the oligonucleotide probes is to incorporate digoxigenin or biotin-labeled nucleotides at the 3′ end of the probes using the terminal transferase enzyme. This approach is less expensive then the incorporation of amino-modified nucleotides during the synthesis of the probes and can give a higher signal because of the utilization of antibodies for the detection. However, the yield of incorporation of modified nucleotides by the terminal transferase can vary among the probes and quantitative measurements are not possible.

1. Purify the oligonucleotide probes by gel electrophoresis.
2. The reaction mix (50 μl) consists of 25 p*M* oligonucleotide probe, 0.2 m*M* digoxigenin-11-dUTP or biotin-16-dUTP, 1 m*M* $CoCl_2$, 140 m*M* potassium cacodylate, 30 m*M* Tris, pH 7.6, 0.1 m*M* DTT, and 100 U terminal transferase. Incubate at 37° for 1 hr. The terminal transferase and modified dUTP are available from Boehringer-Mannheim (Indianapolis, IN).
3. Purify the probes with two rounds of gel filtration on Micro Bio-Spin columns with Bio-Gel P30 (Bio-Rad, Hercules, CA).

RNA Probes

Short RNA probes (50–200 nucleotides) can also be used for *in situ* hybridization. They have the advantage of being less expensive than oligo-

nucleotides probes. However, they give higher background and necessitate more stringent washing conditions. It is very important to design the RNA probes such that no polylinker sequences are present in the resulting transcript. Indeed, the polylinker often contains GC-rich stretches that induce cross-hybridization with ribosomal RNA.[13] These RNA probes are synthesized *in vitro* using the T7, T3, or SP6 RNA polymerase, and an amino-modified nucleotide [usually amino-allyl(U)] is incorporated during the transcription. *In vitro* transcription kits are available from companies such as Ambion (Austin, TX) and can be adapted for the incorporation of modified nucleotides.

Notes. The T7 and T3 RNA polymerases incorporate amino-modified nucleotides better than the SP6 RNA polymerase. Digoxigenin-11-UTP, biotin-16-UTP, and fluorescein-12-UTP (all from Boehringer-Mannheim, Indianapolis, IN) can also be incorporated using the same protocol. However, fluorophore-labeled UTPs are incorporated at a lower frequency, which result in a lower specific activity for the probes. Also, these transcription reactions cannot be phenol extracted, as the fluorophore-labeled RNA probe partition with the phenolic phase.

Reaction Mix[14]

40 m*M* Tris, pH 7.5
6 m*M* $MgCl_2$
2 m*M* spermidine
10 m*M* NaCl
20 m*M* dithiothreitol
1 m*M* ATP, CTP, GTP, UTP
1 m*M*[15] 5-(3-aminoallyl)UTP (Sigma, St. Louis, MO)
2 U/μl RNase inhibitor
40 ng/μl linearized plasmid DNA
0.2 U/μl RNA polymerase

Complete with DEPC-treated H_2O. Incubate at 37° for 1–2 hr. Add 1 U of DNase/RNase free and incubate at 37° for 10 min. Purify the RNA product by phenol extraction and ethanol precipitation. The RNA is then resuspended in 1× SSC (0.15 *M* NaCl, 15 m*M* sodium citrate, pH 7), and unincorporated nucleotides are removed by two rounds of gel filtration on Micro Bio-Spin columns with Bio-Gel P30 (Bio-Rad, Hercules, CA). The

[13] H. Witkiewick, M. E. Bolander, and D. R. Edwards, *BioTechniques* **14,** 458 (1993).
[14] M. R. Jacobson and T. Pederson, *in* "mRNA Formation and Function," p. 341. Academic Press, San Diego, 1997.
[15] If a high ratio of fluorophore coupling is preferred, it is possible to use only aminoallyl-UTP in the reaction instead of a 1:1 mix of UTP:aminoallyl-UTP.

RNA is finally precipitated with ethanol and resuspended in DEPC-treated water.

Conjugation of Activated Fluorophore to Amino-Modified Probes

Manipulations should be conducted under low luminosity in order to avoid bleaching of the fluorophores. Also, any trace of free primary amine, such as Tris base, should be removed from the nucleic acids, as it will also react with the activated fluorophore.

Dissolve 20 μg of dried, purified, amino-modified oligonucleotide or RNA in 35 μl of 0.1 *M* sodium carbonate buffer, pH 9.0 (use pH 8.8 and shorter incubation time for RNA probes in order to decrease the hydrolysis of the RNA during the incubation). Resuspend the activated fluorophore in 15 μl dimethyl sulfoxide (DMSO) and add to the oligonucleotide solution (CY3 is water soluble and does not require DMSO). Incubate for 12–16 hr for oligonucleotides and 6–12 hr for RNA probes, in the dark, at room temperature, with occasional vortexing. Commonly used fluorophores are CY3 (Amersham, Arlington Heights, IL) and rhodamine (Molecular Probes, Eugene, OR) as red fluorophores and FITC and Oregon Green 488[16] (both from Molecular Probes, Eugene, OR) as green fluorophores.

Unreacted fluorophores are removed by gel filtration through a Sephadex G-50 column. Fractions containing the labeled oligonucleotide are visualized under a UV lamp and pooled. The specific activity of the probe is calculated by absorption spectroscopy. If several probes are used to detect a single RNA, they can be pooled at a concentration of 1 ng/μl for each probe and stored at −20° in the dark.

In Situ Hybridization on Yeast Cells

At this step, it is important that all solutions for fixation, spheroplasting, and *in situ* hybridization be DEPC-treated or prepared with DEPC-treated distilled water. Also, individuals must wear gloves to avoid RNase contaminations. This protocol is derived from Long and colleagues.[17]

Preparation of Coverslips

To maintain yeast cells at the surface of the coverslip during hybridization and washing steps, the coverslips must be coated with poly(L-lysine).

[16] The coupling of Oregon Green 488 to amino-modified nucleotides is very slow and an incubation time of 48 hr is preferable in this case. We usually do not use this fluorophore with RNA probes because of the higher degradation level of the probes.

[17] R. M Long, D. J. Elliot, F. Stutz, M. Rosbash, and R. H. Singer, *RNA* **1,** 1071 (1995).

Type 1 coverslips (22 × 22 mm, Fisher Scientific, Pittsburgh, PA) are first boiled in 250 ml of 0.1 *N* HCl for 30 min. They are rinsed 10 times with distilled water in a beaker and autoclaved in distilled water. They can be stored at 4° for several months.

Put one coverslip in each well of a six-well tissue culture plate (Becton-Dickinson, Franklin Lakes, NJ) and drop 200 μl of poly(L-lysine) 0.01% (Sigma) on each coverslip. Incubate for 2 min at room temperature, aspirate the excess, and let dry at room temperature (about 2–3 hr). When dry, wash each well three times for 10 min with distilled water, which is removed by aspiration. At the third wash, rest each coverslip on the wall of the wells, with the face treated with poly(L-lysine) on the top, and let dry (do not let the coverslips air dry on the bottom of the wells as they will stick to the plastic).

Fixation of Yeast Cells

Yeasts are grown in 50-ml cultures in the appropriate media until they reach early log phase (OD_{600} between 0.2 and 0.4, about 10^8 cells). Cells are fixed for 45 min at room temperature by directly adding 6.3 ml of 32% formaldehyde (EM grade, Electron Microscopy Sciences, Fort Washington, PA) to the medium. The fixative is removed by three rounds of centrifugation (5 min at 3500 rpm at 4°) and washes with 10 ml of ice-cold buffer B (1.2 *M* sorbitol, 0.1 *M* potassium phosphate, pH 7.5).

Notes. For the detection of nuclear RNA, one can use 10 ml of 20% formaldehyde, 50% acetic acid for 10 min. The quality of the formaldehyde is crucial for the preservation and detection of small details by FISH. We strongly suggest using ultrapure, single-usage ampoule-sealed formaldehyde. If not possible, a fresh solution of 40% paraformaldehyde can be prepared by adding 12 g of paraformaldehyde (Sigma) to 22.8 ml of DEPC-treated water with 6 drops of 10 *N* NaOH.

Spheroplasting

Cells are resuspended (do not vortex) in 1 ml of buffer B containing 20 m*M* vanadylribonucleoside complex (Gibco-BRL, Gaithersburg, MD), 28 m*M* 2-mercaptoethanol, 0.06 mg/ml phenylmethylsulfonyl fluoride, 5 μg/ml of pepstatin, 5 μg/ml of leupeptin, 5 μg/ml of aprotinin (all from Sigma), and 120 U/ml of RNase inhibitor (Boehringer-Mannheim, Indianapolis, IN) and transferred in a tube containing 0.1 mg of dried oxalyticase (50,000 U/mg, Enzogenetics, Corvallis, OR). Spheroplasting is done by incubating the cells for 8 min at 30° (up to 15 min for strains with more resistant walls). Cells are then centrifuged for 4 min at 3500 rpm at 4° and washed once in ice-cold buffer B. Cells are further resuspended in 750 μl

of buffer B, and 100 μl is added to each of the poly(L-lysine)-coated coverslips in the six-well tissue culture plates. Cells are left to adhere to the coverslips by incubating for 30 min at 4°. Three milliliters of buffer B is then carefully added to each well, removed by suction, and replaced by 5 ml of 70% (v/v) ethanol, which is incubated for at least 15 min at −20° before hybridization. At this stage, the coverslips can be stored a few weeks at −20°.

Note. The oxalyticase should be resuspended in 50 m*M* potassium phosphate, pH 7.5, at 1 mg/ml, aliquoted at 100 μl (0.1 mg) per tube, and lyophilized. These aliquots can be stored at 4° in a dessicator. To test if the spheroplasting went well, drop 20 μl of 1% SDS on 100 μl of spheroplasted cells on a glass slide and mix. Spheroplasts will blow out and a white, flaky precipitate should appear.

In Situ Hybridization

All the following steps should be done under low luminosity in order to avoid the bleaching of the fluorophores on the probes, especially for fluorescein isothiocyanate (FITC)-labeled probes.

Preparation of Probes. For each coverslip used in the hybridization, prepare one tube of probes (we suggest using two coverslip per experiment in order to have a duplicate if one of the coverslip is broken during the manipulations). Add 10 μl of the 1-ng/μl probe solution with 4 μl of a 5-mg/ml solution of 1:1 sonicated salmon sperm DNA:*E. coli* tRNA (Sigma). Lyophylize and resuspend in 12 μl of 80% formamide (Sigma) and 10 m*M* sodium phosphate, pH 7.0.

Hybridization. The cell-coated coverslips are put in a Coplin jar (Thomas Scientific, Swedesboro, NJ) and rehydrated with two washes of 8 ml of 2× SSC (0.3 *M* NaCl, 30 m*M* sodium citrate, pH 7) for 5 min at room temperature. Incubate coverslips in 8 ml of 40% formamide, 2× SSC for 5 min at room temperature. During this incubation, heat the 12-μl solution of probes at 95° for 3 min and add 12 μl of 4× SSC (0.75 *M* NaCl, 75 m*M* sodium citrate, pH 7), 20 m*M* vanadylribonucleoside complex, 4 μg/μl of RNase-free BSA (Boehringer-Mannheim, Indianapolis, IN), and 50 U of RNase inhibitor. Drop 24 μl of the probe solution on a Parafilm sheet wrapped around a glass plate (16 × 20 cm) and lay the coverslip on the drop (the surface of the coverslip containing the cells should face the drop. Be careful to avoid air bubbles). Up to 10 coverslips can be placed on these plates. Finally, wrap over a second Parafilm sheet (do not move the coverslips after they have been placed), seal the two Parafilm sheets together, wrap the glass plate in aluminum foil, and incubate at 37° for 3 hr to overnight (a 3-hr incubation gives a lower hybridization signal).

Notes. The quality of the formamide is important in order to avoid nonspecific signaling. Keep the formamide stock at 4° and do not store for more than a year. The probes must be resuspended in a solution containing twice the final concentration of formamide, as they will be diluted subsequently. If the hybridization is done against the poly(A) mRNA population with a poly(dT) probe, use a 10% formamide solution instead of 40% at each step of the hybridization. For RNA probes, use 50% formamide.

Washing. After the incubation, remove the coverslips from the Parafilm sheet and put them back in the Coplin jar. Wash the coverslips twice with 8 ml of 40% formamide, 2× SSC (preheated at 37°) for 15 min at 37° (for RNA probes, wash at 50°). Wash with 8 ml of 2× SSC, 0.1% Triton X-100 for 15 min at room temperature and then twice with 8 ml of 1× SSC for 15 min at room temperature. Finally, add 8 ml of 1× PBS (phosphate-buffered saline: 1 m*M* KH_2PO_4, 10 m*M* Na_2HPO_4, 140 m*M* NaCl, 3 m*M* KCl, pH 7.4) containing 1 ng/ml of DAPI (Molecular Probes, Eugene, OR).

At this step, the coverslips are ready to be mounted on the glass slides (1 mm thick, Gold Seal Products, Highland Park, IL). Drop 10 μl of mounting medium (see next paragraph) on the slide, lay down the coverslip on the drop (the surface of the coverslip containing the cells should face the drop), and remove excess medium with Kimwipes. Finally, seal the coverslip sides with nail polish. The slides can be stored at −20° for several months without loss of fluorescence.

Preparation of Mounting Medium. This medium contains *p*-phenylenediamine, which retards the photobleaching of fluorophores. Dissolve 100 mg of *p*-phenylenediamine (Sigma) in 10 ml of 10× PBS (10 m*M* KH_2PO_4, 0.1 *M* Na_2HPO_4, 1.4 *M* NaCl, 40 m*M* KCl, pH 7.5) and adjust to pH 8.0 with 0.5 *M* sodium bicarbonate, pH 9.0 (freshly prepared). Add 90 ml of glycerol and 10 μl of 1 mg/ml DAPI and keep at −20° wrapped in aluminum foil.

Note. We recommend storing in multiple aliquots in order to prevent frequent thawing–freezing of the stock solution. The manipulations should be done in the dark. The color of the solution changes with time, going from yellow to dark brown (which indicates oxidation of the phenylenediamine). Discard if the color is too dark in order to prevent fluorescence artifacts.

In Situ Hybridization on Cultured Cells

Preparation of Coverslips

The coverslips are prepared the same way as in the section on yeast cells. However, if fibroblast cells are used, it is preferable to use gelatin-

coated coverslips instead of poly(L-lysine) because the fibroblasts do not adhere well on poly(L-lysine).

To prepare gelatin-coated coverslip, treat the coverslips with HCl and rinse with distilled water (see *in situ* hybridization on yeast cells). Put the coverslips in a solution of 0.5% gelatin (Fisher Scientific, Pittsburgh, PA) and autoclave. Keep at 4°.

Coverslips are placed in a 100-mm petri dish (Fisher Scientific), and cultured cells are grown directly on the coverslips in the appropriate growth medium at 37°, up to 80% confluence.

Fixation

The cells are washed once in 1× PBS (10 m*M* Na_2HPO_4, 1 m*M* KH_2PO_4, 137 m*M* NaCl, 3 m*M* KCl, pH 7.4) and fixed for 10 min at room temperature in 4% formaldehyde (from a 32% liquid stock, Electron Microscopy Sciences, Eugene, OR), 10% acetic acid, 1× PBS. After two washes with 1× PBS, cells are dehydrated by treatment with 70% ethanol overnight at 4°. Coverslips can be stored for weeks at this stage.

Notes. The presence of acetic acid improves detection of nuclear RNA. It can be replaced by a Triton X-100 extraction prior to fixation. Neither of these treatments is necessary when short (50 bases) oligonucleotide probes are used (a 30-min fixation in 4% formaldehyde, 1× PBS is sufficient in this case) or when one wants to detect cytoplasmic RNAs.

Hybridization

The cells are rehydrated for 5 min, at room temperature, in 2× SSC (0.3 *M* NaCl, 30 m*M* sodium citrate, pH 7.0), 50% formamide. Cells are hybridized overnight at 37° (in the dark) in 40 μl of a solution containing 10% dextran sulfate, 2 m*M* vanadylribonucleoside complex, 0.02% RNase-free BSA, 40 μg *E. coli* tRNA, 2× SSC, 50% formamide, and 30 ng of probe. (See the section on yeast for a detailed description of the manipulations.)

Washing

The cells are washed twice for 30 min at the appropriate stringency: 2× SSC, 50% formamide, 37° for oligonucleotide probes or 0.1× SSC (15 m*M* NaCl, 1.5 m*M* sodium citrate, pH 7.0), 50% formamide, 50° for RNA probes. The coverslips are finally mounted on glass slides with mounting medium (see section on yeast cells) and stored at −20°.

Note. Probes that are labeled with high specific activity tend to give higher background, but the addition of 0.1% Nonidet P-40 (NP-40) or 0.1% SDS in the washing buffer can diminish this background.

Simultaneous in Situ Hybridization and Immunofluorescence (Optional)

This protocol can be used if the simultaneous detection of RNA and protein is required in both yeast[10] and cultured cells.[18] It can also be used for the detection of digoxigenin-labeled probes using antidigoxigenin antibodies.[19] In both cases, the *in situ* hybridization has to be performed first, followed by immunofluorescence.

Primary Antibody. Following the last wash with 1× SSC, the coverslips are incubated in 8 ml of 1× PBS, 0.1% BSA, in 8 ml of 1× PBS, 0.1% BSA, 0.1% NP-40, and in 8 ml 1× PBS, 0.1% BSA, all for 5 min at room temperature. The primary antibody is diluted at the appropriate concentration in 1× PBS, 0.1% BSA, 20 m*M* vanadylribonucleoside complex, and 120 U/ml of RNase inhibitor. Drop 25 μl of antibody solution on glass plates covered by a Parafilm sheet and lay the coverslips on the drops. Cover with a second sheet of Parafilm, seal, and wrap with aluminum foil. Incubate at 37° for 1–2 hr in the dark.

Notes. The 1× PBS, 0.1% BSA solution should be prepared fresh from a 10× PBS DEPC-treated solution and molecular biology grade BSA (Boehringer-Mannheim). The final solution should be filter-sterilized and not autoclaved. Use Triton X-100 instead of NP-40 for mammalian cells. For the detection of digoxigenin-labeled probes, antibodies are available from Boehringer-Mannheim.

Washing. Transfer the coverslips in the Coplin jar and wash sequentially for 15 min at room temperature with 8 ml of 1× PBS, 0.1% BSA, followed by 8 ml of 1× PBS, 0.1% BSA, 0.1% NP-40, and finally with 8 ml of 1× PBS, 0.1% BSA.

Secondary Antibody. The secondary antibody is diluted at the appropriate concentration in 1× PBS, 0.1% BSA, 20 m*M* vanadylribonucleoside complex, and 120 U/ml of RNase inhibitor. The coverslips are incubated in the presence of the secondary antibody for 1 hr at room temperature in the dark.

Notes. Antibodies conjugated to green fluorophores (e.g., FITC, CY2), red fluorophores (e.g., CY3, Rhodamine, and Texas Red), or blue fluorophore (e.g., AMCA) can be purchased from Jackson ImmunoResearch (West Grove, PA).

Washing. Coverslips are subsequently washed once for 15 min at room temperature in 8 ml of 1× PBS, 0.1% BSA, twice in 1× PBS, 0.1% BSA, 0.1% NP-40, and once in 8 ml of 1× PBS, 0.1% BSA. The coverslips are

[18] C. Jolly, F. Mongelard, M. Robert-Nicoud, and C. Vourc'h, *J. Histochem. Cytochem,* **45,** 1585 (1997).

[19] P. A. Takizawa, A. Sill, J. R. Swedlow, I. Herskowitz, and R. D. Vale, *Nature* **389,** 90 (1997).

then rinsed in 1× PBS and 1 ng/ml of DAPI and mounted on slides with mounting medium. They can be stored at −20° for several months.

Microscopy and Imaging

Microscopes

A good microscope is essential for obtaining good quality results from *in situ* hybridizations. Several manufacturers, e.g., Nikon (Melville, NY), Olympus (Melville, NY), and Zeiss (Thornwood, NY), offer different types of microscopes. Of course, state-of-the-art microscopes are not necessary for most of the FISH and immunofluorescence applications in yeast and cultured cells, and equivalent results are obtained with less expensive microscopes with good optics.

A suitable epifluorescence microscope for *in situ* hybridization work should contain a UV lamp, fluorescence excitation and emission filters, a condenser and filters for Nomarski or phase, and a wide-field objective. Because each of these items is usually sold separately from the microscope, it is possible to set the microscope to your particular needs. Two pieces are essential for good quality microscopy: fluorescence filters and objectives. Even if some fluorophores have similar maximum excitation wavelengths, such as Texas Red (595 nm), Rhodamine Red (570 nm), and CY3 (550 nm), it is important to use the appropriate filter for a given fluorochrome in order to have a maximal light emission from this fluorophore. These filters are available from the microscope manufacturers or from specialized suppliers such as Chroma Technology (Brattleboro, VT).

For objectives, magnification lens of 40 to 100× (for a total magnification of 400–600 to 1000–1500× depending on the eyepiece) are usually appropriate for *in situ* hybridization work in both yeast and cultured cells. It is important to note that the brightness of the fluorescence image is inversely proportional to the magnification of the lens. To increase both the intensity of the excitatory light and the emission fluorescence, it is appropriate to use objectives with high numerical aperture (NA) in oil immersion. The numerical aperture value reflects the angle of the cone of light accessible by the objective; a wider cone will emit and gather more photons. Objectives with higher NA also have the advantage of a higher resolving power. Finally, most objectives are corrected for field curvature aberrations and produce a flat field of observation. These objectives range from plan achromat, plan fluorites to plan apochromats, depending on the quality of the correction (and the price). For an accessible and excellent review on light microscopy, we suggest the web site of Molecular Expressions (www.micro.magnet.fsu.edu).

Imaging

After *in situ* hybridizations have been performed, it is important to acquire images of the cells for publication purposes or simply data storage. The main choices for image acquisition technologies are between well-known reflex cameras and charge-coupled device (CCD) cameras. Photomicroscopy with reflex cameras offers a less expensive advantage than CCD cameras (although film costs must be considered). These cameras are usually sold by the microscope manufacturers and use standard black-and-white or color films. Usually, an ASA 400 film is sufficient for good quality images of both cultured and yeast[20] cells. However, several images of the same cells with different exposure times must be acquired and, depending on the signal intensity, exposure times of several seconds are necessary. These long exposure times will increase the bleaching of the samples. Also, the images captured are nonquantitative and have a smaller dynamic range compared to a CCD camera.

CCD cameras use silicon microchips to detect photons and produce a digital readout that can be stored and processed on a computer. These cameras have a very low noise background and are very sensitive, which makes them useful for the detection of low hybridization signals. Exposure times of 100 msec to a few seconds are usually sufficient. Cooled CCD cameras ranging from 8 to 16 bits can be purchased from companies such as Photometrics (Tucson, AZ), Olympus (Melville, NY), or Hamamatsu (Japan). The number of bits reflects the dynamic range (the ability to detect very dim and very bright parts in a single image) and the gray-scale resolution of the camera. The hardware and software must allow the processing and storage of digital images of a few megabytes, which are usually captured. This means that a computer of 32 MB of RAM and several hundred megabytes for storage is necessary for image acquisition. Long-term storage on an optical disk or CD-ROM with a 650MB capacity is recommended. These images can be restored and processed with software such as Adobe Photoshop (Adobe Systems, Mountain View, CA) or NIH Image (NIH, Bethesda, MD).

Conclusion

The use of chemically defined probes for FISH in conjunction with high-resolution optics, sensitive means of image detection, and sophisticated algorithms to extract three-dimensional quantitative information at super-resolution allows the study of the molecular biology of individual cells using

[20] J. R. Pringle, A. E. M. Adams, D. G. Drubin, and B. K. Haarer, *Methods Enzymol.* **191** (1991).

microscopy. The FISH approach allows the conversion of the nucleic acid sequence into physiologically relevant information. New developments are forthcoming in real-time imaging of specific squences in living cells and in more powerful optics and more extensive analysis of images, which will provide us with increasingly abundant information concerning the expression of nucleic acid sequences within cells.