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SIMULTANEOUS IDENTIFICATION OF A SPECIFIC GENE PROTEIN PRODUCT AND TRANSCRIPT USING COMBINED IMMUNOCYTOCHEMISTRY AND *IN SITU* HYBRIDIZATION WITH NON-RADIOACTIVE PROBES

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Abstract

Simultaneous identification of messenger RNA (mRNA) and proteins in the same cells or tissues is a valuable tool to help the cell biologist evaluate the cell secretory cycle. Some cells may produce the mRNA and delay the production of the proteins. Alternatively, the proteins may be rapidly secreted. Other cells may produce both in sequence within the same time frame. Because of this difference, some cells can only be identified by their mRNA product. Others may have both products. This presentation describes a non-radioactive approach to the detection of both products with dual-peroxidase labeling protocols in use in this laboratory since 1983. The first detection system uses biotinylated cRNA probes or oligoprobes in *in situ* hybridization along with antisera to biotin to detect the hybrid. The detection system is amplified by 2-3 layers of anti-biotin, second antibody (made against the anti-biotin) and streptavidin conjugated to horseradish peroxidase. After the mRNA is detected with a blue-black substrate (nickel intensified diaminobenzidine), the antigens are detected with immunoperoxidase techniques and orange-amber substrate. The *in situ* hybridization protocol can also be used at the electron microscopic level. Trouble shooting and control protocols are also described. This approach has been shown to be valuable for detection of pituitary hormones, growth factors mRNAs and antigens.

Key Words: *In situ* hybridization, immunoperoxidase cytochemistry, messenger RNA (mRNA), cRNA, oligoprobe, avidin-biotin cytochemistry, pituitary hormones, electron microscopy

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Introduction

Hybridization refers to the reaction between two single-stranded nucleic acid molecules that bind by means of hydrogen bonding of complementary base pairs. Hybridization performed "*in situ*" refers to those techniques that allow binding and detection of hybrids in a cell or tissue section.

Initially, *in situ* hybridization was performed to detect deoxyribonucleic acid (DNA) targets or amplified ribosomal ribonucleic acid (RNA genes) within cell nuclei (Gall and Pardue, 1969; John *et al.*, 1969; Buorgiorno-Nardelli and Amaldi, 1970). Early researchers and clinicians have also mapped the location of genes within chromosomal preparations or nuclei (Pardue and Dawid, 1981; Fostel *et al.*, 1984). The early uses of *in situ* hybridization for cytoplasmic RNA involved detection of viral nucleic acid sequences within infected tissues (Brahic and Haase, 1978; Haase *et al.*, 1981).

Early investigators used radiolabeled complementary cDNA or cRNA probes for the hybridization reaction. Detection of the hybrids then involved autoradiography. However, during the past decade other detection systems have developed that can be as efficient as those with radioactive labels and take only a day or two to perform. Most of these involve the attachment of a signaling hapten molecule such as biotin or digoxygenin to the cDNA or cRNA probe. The detection systems for these molecules are enhanced by sandwich techniques that apply different layers of reactants, or enzyme reactions, or both. The techniques can be readily applied to the detection of both mRNA and antigens within the cells (by dual-labeling approaches).

The purpose of this presentation will be to describe a method for the detection of mRNAs and antigens with dual-labeling immunocytochemistry (Childs *et al.*, 1987, 1990, 1991a,b, 1994; Kaiser *et al.*, 1992; Lee *et al.*, 1993; Fan *et al.*, 1995). We use biotinylated complementary oligonucleotide or cRNA probes to hybridize with the cytoplasmic mRNAs. Then we detect the probes with anti-biotin and enhance the reaction by a sandwich technique that provides layers of biotin and streptavidin-peroxidase (McQuaid and Allan, 1992; Fan

et al., 1995). After the initial reaction, we can detect the pituitary antigens by classical immunoperoxidase cytochemistry with the use of a different colored substrate (Childs *et al.*, 1987, 1990, 1991a,b, 1994; Kaiser *et al.*, 1992; Lee *et al.*, 1993).

Materials and Methods

When we first detected mRNA hybrids in whole pituitary cells, we used the avidin biotin peroxidase complex technique (ABC *Elite*, Vector Laboratories, Burlingame, CA) (Childs *et al.*, 1987, 1990, 1991a,b, 1992a,b, 1994; Kaiser *et al.*, 1992; Lee *et al.*, 1993). We no longer use this complex to detect the biotinylated probes because we frequently encountered high background with ABC kits used from 1993 to the present. The early *Elite* kits did not have these background problems. Thus, we now use a new five-step immunolabeling protocol that detects biotin conjugated to the cRNA probes with anti-biotin. It was first described by McQuaid and Allan (1992). This protocol will be described in the following paragraphs.

Preparation of pituitary cells

To maximize our ability to detect mRNA and antigens, the protocol is applied to cells plated for 1-36 h on 13 mm glass coverslips (Thomas Scientific, Suresdesboro, NJ; Catalog number 6672A75) in 24 well trays (Fisher Scientific, Houston, TX; Catalog number 08-757-156). The protocol can also be used on frozen sections or paraffin sections. The probes used for the hybridization are either cRNA probes (Childs *et al.*, 1987, 1990, 1991a,b; Fan *et al.*, 1995) or complementary oligonucleotide probes at least 30 mer long (Childs *et al.*, 1992 a,b, 1994; Kaiser *et al.*, 1992; Lee *et al.*, 1993). They may be biotinylated by the Vector Photobiotin kit (Vector Laboratories) following kit instructions (Childs *et al.*, 1987; Wu *et al.*, 1991, 1992). More recently, we have ordered the oligonucleotide probes commercially prepared, with Biotin attached. Alternatively, we add Biotin-UTP to the cRNA probes produced during in vitro transcription (Fan *et al.*, 1995).

All fixation, washing, and handling methods are run under sterile conditions to prevent RNase contamination. The buffer or diluent solutions are made, Millipore filtered, and stored frozen in small aliquots. Controls include substitution of labeled sense sequences, or omission of the labeled probe. The protocol can be adapted for use with dispersed cells at the electron microscopic level.

Preparation of solutions

The following list includes the solutions needed for *in situ* hybridization. Table 1 provides information about some of the major vendors that supply these reagents.

(1) Phosphate buffered saline (PBS): 0.1 M phosphate buffer + 0.9% NaCl pH 7.2

(2) Triton X-100 (0.3%): 300 µl Triton X-100 (Sigma Chemical, St. Louis, MO); bring to 100 ml with 0.1 M PBS.

(3) EDTA (50 mM): add 1.46 g EDTA to 100 ml of 0.1 M Tris buffer (Sigma Chemical, Catalog number T-5030) (20 ml 0.5 M Tris + 80 ml Millipore-filtered water), pH 8.0.

(4) Para-formaldehyde (4%): 4.0 g para-formaldehyde in 100 ml 0.1 M PBS, pH 7.2.

(5) Acetic anhydride (0.25%) + 0.1M triethanolamine: 250 µl acetic anhydride; bring to 100 ml with Millipore filtered water, then add 1.856 g triethanolamine, pH 8.0 (Sigma Chemical)

(6) Deionized formamide (50%) in 2X Sodium Salt Citrate (SSC): make a 1:10 dilution from 20X SSC with Millipore-filtered water and add 1:1 formamide (Sigma Chemical, Catalog number F-7503)

(7) Sodium Salt Citrate Stock (SSC): make 20X SSC stock by adding 3 M sodium chloride + 0.3 M Sodium Citrate to Millipore filtered water. Make 4X solution with a 1:5 dilution from stock 20X SSC with Millipore filtered water

Another task is to prepare the *in situ* hybridization buffer. The components are added in sequence in the following instructions. It usually takes about a day and it can be prepared in advance. Most of these components come from Sigma Chemical, unless otherwise noted (see Table 1).

(1) Deionize formamide: add 1.5 g REXYN I-300 (Fisher Scientific, Catalog number R-208) to 100 ml formamide, stir for 45-60 min, filter through Whatman filter paper.

(2) Combine 100 ml deionized formamide + 100 ml 4X SSC (20 ml 20X SSC + 80 ml Millipore filtered water). The final concentration will be 50% formamide.

(3) Add 7.88 g Tris salt (0.25 M), pH 7.5, warm to about 37°C in 1.0 ml Millipore filtered water. Dissolve Tris.

(4) With sterile syringe and needle, add the following to the warm formamide-Tris solution: 20 g dextran sulfate; 0.5 g bovine serum albumin (RIA grade) (Sigma Chemical, Catalog number A-7638), 0.5 g Ficoll-400, 0.5 g Polyvinyl pyrrolidone-360, 1.0 g sodium pyrophosphate, 1.0 g sodium lauryl sulfate.

(5) Finally, add 200 µl of dissolved salmon sperm DNA (ssDNA) to 200 ml of hybridization buffer.

(6) Aliquot into 10 ml units and store frozen (-20°C) in 15 ml centrifuge tubes

Prehybridization protocol

The next series of steps involve preparing the cells for the hybridization. Several components are used to

Table 1. Vendors for *in situ* hybridization histochemistry, including Uniform Resource Locator (URL, Internet link)

Vendor	Address	Telephone number	Internet
DAKO Corporation	6392 Via Real, Carpenteria, CA 93013	800-235-5763	Dimensions has a web page devoted to DAKO Corporation (http://www2.multinet.net/dli/dako.htm) which has access to data sheets
Fisher Scientific	10700 Rockley Road, Houston, TX 77099	800-876-1900 800-766-7000	http://www.fisher1.com
JRH Biosciences	P.O.Box 14848, Lenexa, KS 66325	800-255-6032	
Sigma Chemical	P.O.Box 14508; St. Louis, MO 63178	800-325-3010	http://www.sigma.sial.com/sigma/sigma.html
Thomas Scientific	99 High Hill Road, P.O.Box 99, Suresdesboro, NJ 08085-0099	800-345-2100	
Vector Laboratories	30 Ingold Road, Burlingame, CA 94010	800-227-6666	http://www2.multinet.net/dli/vector.htm

Table 2. Internet Uniform Resource Locator (URL) links to courses, protocols and services in *in situ* hybridization

(1) Link to our protocols including the colored versions of Fig. 1 and other photographs. We will continue to update this page as we progress. URL: http://cellbio.utmb.edu/childs/in_situ.htm

(2) The Anderson Laboratory in situ protocol (URL: http://www.cco.caltech.e...r/htmls/Big_In_situ.html)describes non-radioactive methods for labeling frozen sections, whole mount embryos and cultured cells.

(3) This protocol (URL: http://cellbio.ucdavis.e...mePage/Tucker/WCRDB.html describes single cell RT-PCR and quantitative *in situ* hybridization for beginners.

(4) Dr. Jan Blancato (ONCOR, Inc.) gives a course in *in situ* hybridization. The URL is: http://www.cua.edu/www/catc/ish.htm

(5) Exon-Intron, Inc. runs a course in *in situ* hybridization. The URL is: http://www.dnatech.com/insitu.htm.

(6) Paul Hough at Brookhaven National Laboratory runs an advanced *in situ* hybridization course. The URL is http://www.cshl.org/meetings/96situ.htm.

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aid penetration and several steps are used to prevent non-specific reactions. In our protocol, the pituitary cells are plated on glass coverslips and grown in Dulbecco's Modified Eagle's Medium (DMEM, JRH Biosciences, Lenexa, KS; Catalog number 56499-10L), and fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer) for 30 min at room temperature. Then cells are washed for 1 h in 4 changes of phosphate buffer + 4.5% sucrose. We work under sterile conditions even during the fixation process and store the cells no longer than 1 week at 2-4°C. The steps are taken to prevent RNase contamination which would eliminate RNA from the tissue or cells.

On the day of the hybridization, we first rinse the fixed cells on coverlips with fresh, sterile 0.1 M phosphate buffered saline (PBS) for 5 min at room temperature, shaking. Then we treat in the following sequence of solutions. (All of the following chemicals but the p-formaldehyde came from Sigma Chemical). All steps are done at room temperature.

(1) To aid penetration of reagents, the cells are first treated with 0.3% Triton X-100 for 15 min at room temperature. This is followed by washing with 0.1M PBS (twice, 3 min each, while gently shaking on a side-to side shaker).

(2) To further improve penetration and remove associated proteins, we next treat with Proteinase K (1 μg/ml) for 15 min at room temperature.

(3) The cells are then stabilized by postfixation with 4% para-formaldehyde/0.1M PBS for 5 min at room temperature. This is followed by washing with 0.1 M PBS (twice, 3 min each, while gently shaking).

(4) To cover non-specific reactive sites, we next treat the cells with 0.25% acetic anhydride for 10 min while shaking.

(5) We then treat the cells with 50% formamide/2X SSC for 10 min while shaking the tray at room temperature. Then we continue the incubation at 37°C for 10 min. This solution prepares the cells for the hybridization buffer and helps break weak hydrogen bonds in the non-specific linkages between the complementary probe and the surrounding tissue. If there is a 2-3% mismatch in the complementary probe, the concentration of this solution should be lowered to 40-45% to prevent breaking bonds in the specific probe-mRNA hybrid. This lower concentration should also be used to make the hybridization buffer. (Note that the concentration in the buffer is 50% in the above formulation).

Alternative pre-hybridization technique

We are currently testing pepsin digestion in lieu of the proteinase K and have had successful results with our cells fixed in either glutaraldehyde or para formaldehyde. The Pepsin is purchased from Sigma (Catalog number PN6887) and is made up just before use. Two mg of pepsin are added per ml of 0.01 N HCl. It is stored at room temperature for no longer than two hours. It is added to the cells for 25 min at 37°C followed by an additional 10 min at room temperature.

Hybridization conditions

The cells are then placed in the hybridization solutions which is 300 ng/ml of biotinylated oligonucleotide or cRNA-probe diluted in hybridization buffer (see above). They are incubated for 12-15 h at 37°C. The temperature of hybridization may be varied according to the melting temperature of the probe. We have found that a range from 36 to 42 C works for most of our probes against gonadotropins (Childs *et al.*, 1987, 1992 a,b) or pro-opiomelanotropin mRNAs (Wu *et al.*, 1992).

Post-hybridization protocol

After the overnight hybridization the post-hybridization washing steps are followed. We also add an RNase treatment step if the probe used was a cRNA (Childs *et al.*, 1987; Fan *et al.*, 1995). The coverslips are first washed with 4X SSC (three times, 15 min each; this is done at room temperature with gentle shaking during the last 5 min). This wash is at low enough stringency (high salt concentration) to remove the excess unreacted probe without removing the probe that has specifically hybridized to the mRNA. If there is background with the reaction, higher stringency washes can be done. Also, one can raise the temperature of the washes. This involves continuing the washes with reduced concentrations of SSC (2X, 1X and 0.5X), 15 min each wash. However, we have found this to be unnecessary, especially with our oligonucleotide probes.

Detection of the biotin

After the post-hybridization washes, the detection protocol can be followed.

The detection protocol begins with a blocking step in a solution containing proteins. They cover non-specific sites and prevent background reactions, but they do not react with any of the components of the detection protocol. Therefore, we first block with 0.05 M Tris buffered saline containing 1% bovine serum albumin + 10% normal horse serum (Vector Laboratories) for 15 min at room temperature (pH 7.6). Then we detect the biotin on the hybrids with the following sequence:

(1) The cells are first treated with monoclonal anti-biotin (1:30) (Dako Corp.) for 30 min at 37°C. They are then washed with 0.05 M Tris buffered saline (twice, for 3 min each), pH 7.6).

(2) The cells are then incubated with biotinylated horse anti-mouse IgG (rat absorbed, Vector Laboratories), 1:100, for 10 min at room temperature. They are then washed with 0.05 M Tris buffered saline (twice, 3 min each). It is very important to use rat-absorbed anti-mouse IgG because most of these antisera will react with rat tissues, non-specifically.

(3) The cells are incubated with a second layer of monoclonal Anti-Biotin (1:100) for 30 min at 37°C. Coverslips are then washed with 0.05 M Tris buffered saline (twice, 3 min each).

(4) The cells are incubated with a second layer of biotinylated horse anti-mouse IgG (rat absorbed) 1:100 for 10 min at room temperature and washed with 0.05 M Tris buffered saline (twice, 3 min each).

(5) Finally, we incubate the cells with a 1:10 dilution of peroxidase conjugated streptavidin (Dako, Catalog number P-397) for 5 min at room temperature. The cells are then washed with 0.05 M Tris buffered saline (twice).

At this point, the nickel-intensified diaminobenzidine (DAB) is prepared by dissolving 0.45 g nickel ammonium sulfate in 30 ml 0.05 M acetate buffer, adding 1 diaminobenzidine tablet (Sigma Chemical, Catalog number D-5905). The solution is dissolved. Then we add 20 μl of 30% hydrogen peroxide. The solution is filtered on a Whatman filter paper and then added to the cells for 6 min. The reaction product is blue-black. This reaction cannot be improved by longer times. There is a limited life and time-span of this solution. Therefore, to increase signal, we vary concentrations of the probe or other components of the detection protocol (see

Figure 1. A cluster of pituitary cells, one of which is dual-labeled for FSH-beta mRNA (black, arrows) and LH-beta antigens (gray, L). Note that the label for mRNA is in a linear pattern. The gray label for the LH antigens detects them throughout the cell except on the nucleus. Bar = 10 μm. Reproduced with permission from Childs *et al.* (1994).

Discussion).

After the diaminobenzidine step, the coverslips are washed with 0.05 M acetate buffer (twice), dehydrated, dried and mounted to glass slides, cell side up with Permount. A second square coverslip is mounted over the cells. Note: This DAB reaction product is dissolved in water soluble solutions and therefore cannot be used with water soluble mounting media or glycerol. Therefore, use only organic solvent soluble mounting media with it. One can view the slides for a brief period mounted in glycerol, however eventually this will remove the reaction product.

After the mRNA is detected with the blue-black peroxidase substrate, the cell can be further labeled by immunocytochemistry for its protein or antigen content. For this we use the dual-labeling protocol with contrasting color peroxidase substrates.

Applications of this technique to electron microscopic preparations

We apply the above technique to the detection of mRNA at the electron microscopic level by leaving the dissociated pituitary cells in suspension (Childs *et al.*, 1990). Then, we spin the cells down at 900 rpm after each step. The gentle spinning is effective and creates a pellet which must be resuspended in the new solution. After the diaminobenzidine step, the cells are exposed to 1% osmium tetroxide for 30 min at refrigerator temperatures (2-4°C). This fixes the membrane lipids and enhances the peroxidase reaction. The cell pellets are then embedded in Epon according to traditional electron microscopic methods.

Dual-labeling for antigens

After biotinylated ligands, mRNA or a first antigen is detected we use contrasting colored substrates and immunocytochemistry to detect an antigen (e.g., pituitary hormone, growth factor, or *c-fos*.) The following shows an example of the protocol that detects the antigen. This allows us to identify the hormone content of the cell that expresses the mRNA. The basic dual-labeling techniques have been published since 1983 (Childs *et al.*, 1983). They are outlined below.

(1) Rinse coverslips with 0.05M Tris buffered saline (once).

(2) Block again with 0.05 M Tris buffered saline + 1% bovine serum albumin (Sigma Chemical, Catalog number A-7638) for 15 min at room temperature.

(3) Incubate with specific antisera to antigen in question (diluted 1:5000—1:50,000) for 30 min at 37°C. Wash coverslips with 0.05 M Tris buffered saline (three times).

(4) Incubate with biotinylated goat anti-rabbit IgG (Vector Laboratories, Catalog number BA-1000) (This is made with 25 μl stock Biotinylated-IgG + 25 μl normal goat serum in 2 ml of buffer). Incubate for 20 min at room temperature. Wash coverslips with 0.05 M Tris buffered saline (twice).

(5) Incubate with 1:200 peroxidase conjugated streptavidin (DAKO, Catalog number P-397) for 20 min at room temperature. Wash coverslips with 0.05 M Tris buffered saline (twice).

(6) To detect peroxidase, prepare orange-amber diaminobenzidine. Dissolve 1 Tris buffer tablet in 15 ml Millipore filtered water, add 1 diaminobenzidine tablet (Sigma Chemical, Catalog number D-5905) + 12 μl 30% hydrogen peroxide; filter on Whatman filter paper and use immediately. Apply to cells for 5-7 min at room temperature. Wash coverslips with Millipore filtered water (three times). Dehydrate, dry and mount on slides with Permount.

Note: diaminobenzidine is eventually washed out in water soluble mounting media, especially temporary mounting media like glycerol. Therefore, if another peroxidase substrate is used, that requires water soluble media, please store the slides dry and use glycerol ONLY for short periods of time. The best way to store the slides is to dehydrate the tissues and use permount.

Table 2 lists Internet Uniform Resource Locator

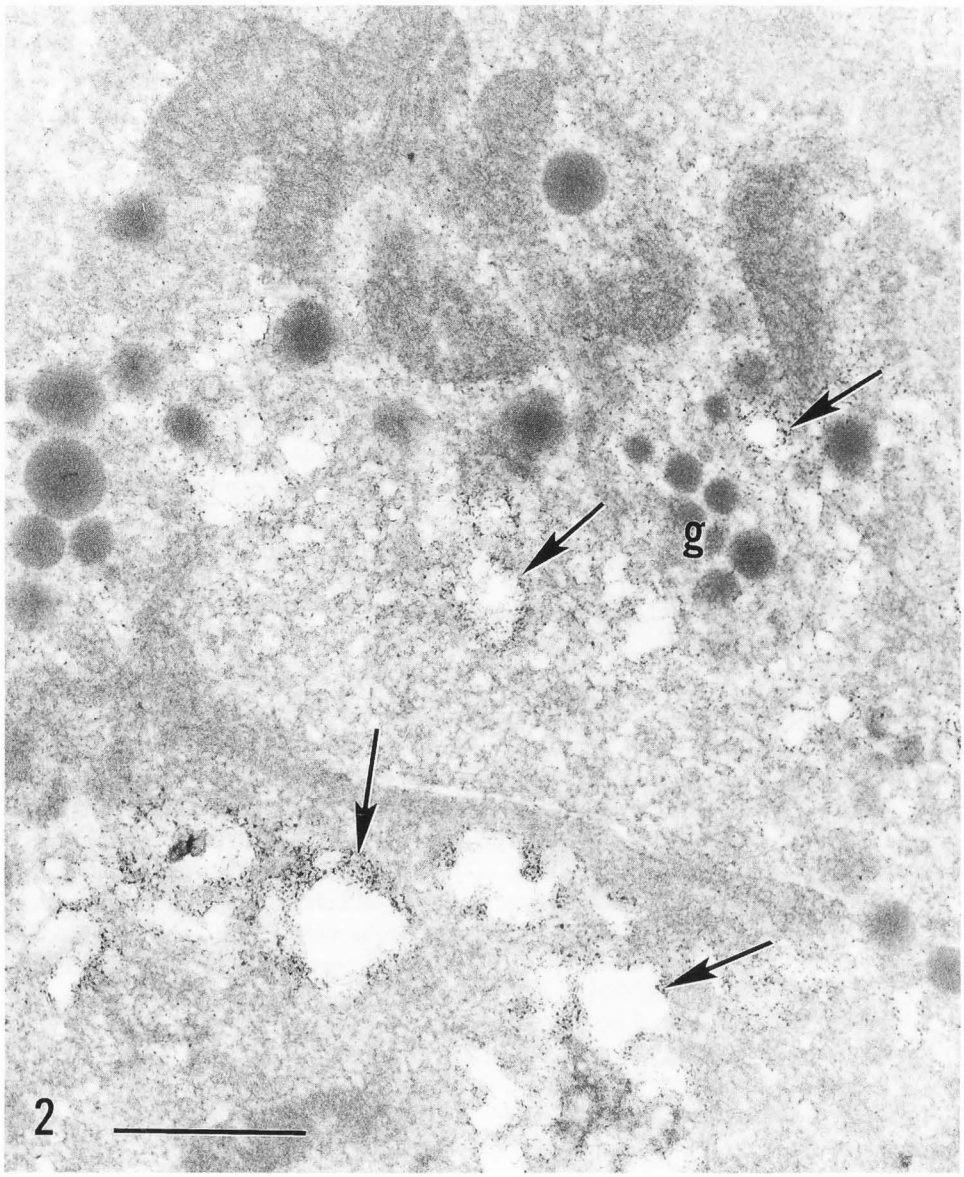

Figure 2. Label for LH-beta mRNA on the surface of rough endoplasmic reticulum detected with a biotinylated cRNA probe and avidin peroxidase (arrows). Note that the secretory granules (g) remain unlabeled. Bar = 0.25 μm.

(URL) links to courses, protocols and services in *in situ* hybridization.

Results and Discussion

The labeling for the mRNA is in dark patches or lines in the specific cell type. This can be confirmed by immunolabeling for the antigen in the same cell (Childs, 1987, 1991a,b, 1992 a,b, 1994; Kaiser *et al.*, 1992; Lee *et al.*, 1993; Fan *et al.*, 1995). However, there are clearly cells that contain only the antigen or only the mRNA (Childs *et al.*, 1994). Thus, we have discovered cells in different stages of their secretory cycle (Childs *et al.*, 1994).

Fig. 1 shows dual labeling for FSH mRNA and LH antigens (Childs *et al.*, 1994). The cell in this figure expresses FSH mRNA in a black linear pattern, and Luteinizing hormone (LH) antigens, labeled orange. In black and white, the orange label is gray and fills the cell.

Fig. 2 shows that, at the electron microscopic level, the label is associated with dilated profiles of rough endoplasmic reticulum (Childs *et al.*, 1990). This field shows labeling for Luteinizing hormone beta subunit mRNA on dilated rough endoplasmic reticulum (RER) in a pituitary gonadotrope. Studies from our laboratory

(Childs *et al.*, 1990) show similar patterns of labeling for Follicle stimulating hormone mRNA.

Troubleshooting this protocol

The label is easy to detect in cells because of its density and linear or patchy pattern. However, it should not be mistaken for the metallic deposits sometimes seen in the cells (pseudoperoxidase activity). The label in cells that do not have amber label should be blue-black or a purplish blue. A steel-gray background over all the cells indicates background problems that should be eliminated (see below).

The *in situ* hybridization fields should always be quantified. Furthermore comparisons should be made between single and dual-labeled preparations. One should count the percentages of labeled cells in fields exposed only to the single labeling protocol After one is confident of the percentages, one should then count the percentages of mRNA-bearing cells in the dual-labeling protocol. They should match. To facilitate this comparison, we have developed a Lotus 1,2,3 or Exel Spreadsheet that automatically calculates these percentages after we load in the raw counts (Childs *et al.*, 1991 a,b, 1994; Lee *et al.*, 1993). We also compare percentages of antigen bearing cells with those obtained through single labeling. These tests are vital to insure that the dual-labeling protocol does not interfere or add to the detected label for either mRNA or antigens. Controls run with the labeling protocol (see below) will also help test the specificity of the reaction.

The labeling density for the mRNA (sensitivity) can be improved by adding another layer of anti-biotin and streptavidin. However, one runs the risk of higher background (McQuaid and Allan, 1992). Also, tests of different temperatures of hybridization may show an optimal temperature that promotes labeling. Finally, one can vary the concentration of probe from 0.1-100 ng/ml for example and the reaction should increase with increasing concentration (up to a plateau point).

cRNA probes usually are more sensitive because they carry more biotin molecules/probe and they are longer. Yet, they are sticky and difficult to work with. They also require expertise in molecular biology techniques for their production. Oligonucleotide probes may carry only one biotin although they can be engineered to carry more. Their sensitivity may also vary with the size and number of biotin molecules. Some low abundance mRNAs may require cRNA probes for adequate detection. Nevertheless, for most of our studies, biotinylated oligonucleotide probes label expected populations of cells at concentrations of 1-100 ng/ml. One could also engineer several biotinylated probes directed against different parts of the mRNA transcript. This may increase sensitivity. All probes must react with unique sequences, however, or specificity will be compromised.

In our hands, this technique does not give high background. Usually the background is crystal clear and the unlabeled cells are difficult to see without the benefit of special optics. However, if there is background, it is usually seen as a steel gray (or light blue) deposit all over the field or cells. Also, it could be seen in dual-labeled fields as light amber label all over the cells. In that case, one might suspect the second labeling protocol. However, both could be at fault. And, both should be checked.

There are several ways to reduce background. First, one can remove one layer of the detection system for the mRNA (an anti-biotin and a anti-mouse IgG step) or dilute the components further. This should be done only if the signal is strong, however. Second, one can increase SSC washes, reducing the concentration to 0.1X. This low salt provides high stringency conditions needed to wash out the non-specifically bound probes that may cause the background. This is especially a problem with the sticky cRNA probes. Caution must be given, however, to the possibility that the washes will wash out the probe attached in the specific hybrids. Finally, the use of different concentrations of the detecting reagents for the biotin (anti-biotin and streptavidin-peroxidase) will reduce background. If the signal is strong, one can dilute both and achieve optimal results.

Controls

We have controlled for interference during the dual labeling by eliminating either the complementary RNA probe from the first sequence or the specific antibody from the second sequence. In each case, the resulting labeling reflects only that which had the complete sequence. Thus, we have proved that the first detection protocol does not add to the label in the second (and visa versa). Furthermore, we have absorbed the specific antibody in the second sequence with its specific antigens. This has eliminated all second reactions and only the blue-black label for the mRNA is visible. This not only proves the specificity of the labeling, it proves that the amber diaminobenzidine does not cause the blue-black label to change color. In studies since 1983, we have shown that the blue-black diaminobenzidine is stable, as long as the second reaction is not prolonged (Childs *et al.*, 1983). This is why the rapid ABC or DAKO immunoperoxidase kits are so valuable. If immunolabeling requires 48 hour incubation in antisera, one runs the risk of losing all detectable reaction product during the second incubation periods. Thus, immunoperoxidase kits with short incubation times should be chosen.

Summary

To summarize, this protocol has been used successfully for the detection of mRNAs in tissue sections (McQuaid and Allan, 1992) and cell cultures (Fan *et al.*, 1995). It is preferred over the direct avidin detection systems because of its sensitivity and the fact that some avidin-containing kits produce high background. The anti-biotin sandwich method provides flexibility and enhancement potential along with a streptavidin solution that works at neutral pH and gives low background reactions.

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Discussion with Reviewers

Reviewer I: In many protocols, methanol treatment makes a variety of cultured cells sufficiently permeable to the probe. Your prehybridization protocol includes proteinase K treatment "to improve penetration (of probe) and remove associated proteins". Proteinase K is a very active, non-specific endopeptidase, at high concentration capable of degrading proteins into single amino-acids. Isn't the proteinase K treatment too hazardous for epitopes to be detected in the same samples, after the hybridization step?

Author: That is an excellent question. The original technique was for use with cRNA probes to detect LH-beta and FSH-beta mRNAs in rat pituitary cells. These large probes (300- 500 bp) required more intense deproteination. Therefore, we used the Proteinase K effectively to aid penetration. At the same time, we recognized the danger to the antigens to be detected in the immunolabeling protocol. Therefore, we tried fixation in 2% glutaraldehyde for 30 min at room temperature before any of the hybridization steps. This fixative gives much stronger cross-linking than any formalin fixative because the glutaraldehyde has two aldehyde groups that can cross link amino acids (formaldehyde has only one group).

We already knew that the anterior pituitary antigens were preserved well in glutaraldehyde-fixed tissues. Therefore, we were pleased when we discovered, in 1987, that the in situ hybridization protocol with proteinase K did not destroy any of the pituitary antigens used to date. In fact, the major result was increased efficiency in the reactions. All primary antisera dilutions were increased at least 2-fold. For example, in a recent experiment with Growth hormone detection, the optimal dilution of anti-GH after dual immunolabeling (two antigens) was 1:20,000-1:40,000. However, after *in situ* hybridization, the optimal dilution of anti-GH was 1:70,000-1:80,000. These antigens are stored in granules and dilated rough endoplasmic reticulum and this may also be a factor in their recovery. Cytoplasmic antigens may be more labile and require less intense deproteination.

Reviewer I: You have discussed reasons for high background associated with detection system for non-radiolabeled probes. However, regardless of the probe labeling method, radioactive or non-radioactive, lack of probe specificity remains one of them. In our hands (35S labeled riboprobes), the most effective way to improve signal to noise ratio, was to use a probe that in Northern analysis would detect a single band of a size matching the expected mRNA size. If this condition was fulfilled, the RN-ase treatment was found unnecessary. Do you think that evaluating specificity of biotin-labeled probes by Northern hybridization might be worth while?
Author: Yes, all of our biotin-labeled cRNA probes (LH, FSH, follistatin, EGF) have been evaluated by either Northern Hybridization or RNase protection assay for specificity. They detected a single band. They were used to assay pituitary mRNAs in collaborative studies.

Reviewer II: The author states that non-radioactive detection systems can be as sensitive as autoradiographic techniques with radiolabeled probes. How can one determine the sensitivity of the hybridization reaction with biotinylated probes?
Author: Actually, these techniques are not purely quantitative in that they do not allow one to detect amounts of DNA or mRNA in a cell. Thus, "efficient" is the operative word, rather than sensitivity. With our system, we use concentrations of cRNA probes for detection that are comparable to those used in autoradiographic detection protocols. If the same enzyme techniques are used in an *in situ* PCR reaction, the amplifying effects of the enzyme substrates allow detection of as little as one copy of mRNA per cell.

Reviewer II: How do various fixation conditions affect the sensitivity of the detection method. Is 2.5% glutaraldehyde used for both light and electron microscopy and/or when performing a dual labeling experiment? Doesn't glutaraldehyde fixation abolish or reduce the antigenicity of endogenous proteins when performing a dual labeling experiment for both mRNA and endogenous proteins.?
Author: We always run comparative studies with any new antigen or mRNA and in every case have found increased, or equal detection efficiency when we used glutaraldehyde. This means that we can use lower concentrations of cRNA probes to get the same signal. We have compared results in a variety of fixatives, including para-formaldehyde, Bouin's, 10% formalin and glutaraldehyde. We just completed another comparison between formalin and 2% glutaraldehyde and its effects on the detection of calcium channel mRNA in pituitary cells. The cells fixed in glutaraldehyde are much more intensely labeled. Furthermore, the peptide antigens are held in place by the stronger fixative and we have a much cleaner reaction in glutaraldehyde fixed tissues. Some of the larger glycoprotein antigens can be detected with much higher dilutions of antisera (1:125,000) if para-formaldehyde is used, however the cell fixation is not optimal. Thus, we choose glutaraldehyde and these same antigens are detected with 1:30,000-1:60,000 dilutions of their primary antisera. One trades efficiency (more dilute antisera) for optimal morphology. However, as stated above, we have never encountered loss of efficiency when detecting mRNA in glutaraldehyde-fixed tissues. Each new graduate student is asked to do the same comparison with a new mRNA and the conclusion has always been the same.

Reviewer II: What is the major advantage of using a biotinylated detection method? What is the major disadvantage of using a biotinylated detection method?
Author: The major advantage is the fact that one can achieve signal in a day, after as little as 2 hours of incubation in the cRNA probe. The longest protocols (with oligoprobes) are 1.5 days. It is also easily adapted

to a rapid immunolabeling technique, so in less than two days, one can detect the mRNA and the antigens. Also, no radioactive compounds are required to add to the growing waste. Finally, because the background is so low (absent), one need not view or depict the cells in dark field. Frequently, autoradiograms must be depicted in dark field, because the background grain level is so high in surrounding tissues. Finally, I have seldom seen autoradiograms where the labeling grains are well confined to the cells in question. It is impossible to detect regional labeling in such cells. Since we know that most cells are not filled with mRNA (100%), most of this is artifactual spread. In the case of the non-radioactive systems, one can also get this spread, however, it can be more readily controlled. In the case of peroxidase, the labeling can be confined to the region of the mRNA, where it belongs (Figure 2). There are no real disadvantages to using any non-radioactive detection system, since it can be adapted to In situ PCR for the low abundance mRNAs. If one has endogenous biotin, one can use the blocking kits supplied by Vector Laboratories. Also, there are blocking kits and reagents available for the enzymes in the detection systems.

Reviewer II: Assuming that the mRNA is associated with cytoplasmic polyribosomes and/or with RER, what is the advantage of detecting mRNA hybrids at the ultrastructural level? What is the major advantage of being able to detect mRNA at the ultrastructural level, i.e., by electron microscopy.

Author: In the pituitary, one cannot always differentiate the cell types accurately by morphology alone. Thus one needs a detection system that identifies the antigens within the granules and/or the mRNA on the rough endoplasmic reticulum. In the case of poorly differentiated pituitary cells, one might have only polyribosomes and few storage granules. Such cells can only be identified by their content of mRNA for the particular hormone. Such cells are abundant among developing gonadotropes, for example.

Reviewer II: How does one adapt this protocol for detection of mRNA in tissue sections? Are different fixation conditions used?

Author: We have used frozen pituitary sections with biotinylated oligoprobes for AVP. We rapidly froze the small tissue blocks and cut the sections with a cryotome. Then, we fixed them in para-formaldehyde vapors. They were then detected with an avidin detection system. In this case, my student (Dr. Ping Wu) preferred to use streptavidin alkaline phosphatase. This was quite successful and her papers are cited in the reference list. We are now working on adapting the streptavidin alkaline phosphatase technique to a dual-labeling protocol and have had some success with the use of immunoperoxidase as the detection system for the antigens. This protocol can be used as is for either cells in culture or frozen sections.

Reviewer II: Where does one block for endogenous biotin, peroxidase, and/or alkaline phosphatase in the method described.

Author: We have no endogenous biotin. We have been using biotin-avidin detection systems in the pituitary for over 15 years, and have never encountered any evidence of endogenous biotin. However, if it is a problem, one can use the blocking reagents supplied by Vector Laboratories. We also have been using peroxidase detection systems since 1972. We see pseudoperoxidase activity exhibited by red blood cells in our immunolabeling protocols. This can be eliminated by pretreatment with hydrogen peroxide. Interestingly, we never see this activity when running *in situ* hybridization protocols. Thus, something in our sequence must quench it. Our control fields are so crystal clear, it is difficult to find the cells without special optics. With regard to alkaline phosphatase, there are blocking kits and reagents available. However, our recent work has shown that they are unnecessary. A recent inadvertent proof of this happened when my technician accidentally left out the streptavidin alkaline phosphatase in the sequence that detected calcium channel mRNA. The cells were exposed to an extended time in the substrate solution. There was absolutely no reaction in these glutaraldehyde fixed cells. Finally, our current experiments have been run on cells plated on silane coated slides with two wells indented in each slide. One of the wells is always a control, the other receives the biotinylated cRNA probe. We wash these slides in coplin jars, in the same solutions. Each slide is exposed to the same substrate solution. Not only are the controls for the single labeling protocol completely clean (showing no endogenous alkaline phosphatase), but the controls for the dual-labeling protocol remain clean (showing no endogenous peroxidase from the second protocol). The control and experimental fields on the same slide are as different as night and day, even if the experimental fields contain many intensely labeled cells.