

REVIEW

Co-labeling Using In Situ PCR: A Review

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SUMMARY In situ amplification permits the histological localization of low-copy DNA and RNA targets. However, in many instances it would be useful to know the specific phenotype of the target-containing cell or to ascertain the distribution of a different nucleic acid sequence in the same tissue section. This review describes a methodology that allows co-in situ localization of two nucleic acid targets or a DNA/RNA sequence and a protein in paraffin-embedded, formalin-fixed tissue. The key variable for detection of low-copy RNA targets by RT in situ PCR is optimal protease digestion to permit cDNA target-specific incorporation of the reporter nucleotide. This is achieved via inactivation of nonspecific DNA synthesis by overnight DNase digestion. The key variable for immunohistochemical localization of proteins is to determine the effect of protease digestion on the antigen-based signal intensity. Background for DNA targets by in situ hybridization or, for targets present in 1–10 copies per cell, PCR ISH is dependent primarily on probe concentration and the stringency of the post-hybridization wash. Radioactive ³H-labeled nucleotides permit an excellent distinction with colorimetric signals for co-localization, although two distinct chromogens can in many instances allow successful localization of two different targets.

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EVER SINCE the first peer-review paper described in situ detection of PCR-amplified DNA in 1990 and PCR-amplified cDNA in 1992 (Haase et al. 1990; Nuovo et al. 1992), there has been great interest in using these methodologies to elucidate the histological distribution of low-copy nucleic acid targets. Given that the method would be most useful in archival material, which usually is paraffin-embedded and fixed in 10% buffered formalin, much attention has focused on optimizing the conditions for such tissues. This review stresses the optimal conditions necessary for successful amplification of DNA [PCR in situ hybridization (ISH) or in situ PCR in which the labeled nucleotide is directly incorporated into the amplicon; this can be employed only in frozen/fixed tissue and not paraffin-embedded tissue] and RNA (RT in situ PCR) in such archival materials. It is well documented that formalin is the superior fixative for in situ analysis for DNA

and RNA targets (McAllister and Rock 1985; Tournier et al. 1987; Nuovo 1989; Lebargy et al. 1990). The other key point to be stressed here is that background, defined as the presence of a signal in cells that do not contain the target of interest, is by no means unique to in situ PCR protocols. As anyone with hands-on experience in immunohistochemistry or standard ISH can attest, background can be a problem with either of these older methodologies and, unless controlled, can lead to false-positive results. For standard ISH it is well documented that too high a concentration of the labeled probe, too long a hybridization time, or a relatively low-stringency post-hybridization wash can easily produce a signal in cells that do not contain the nucleic acid target sequence of interest (Figure 1) (Nuovo 1997). Another key variable for background with standard ISH is the size of the probe. Oligoprobes (usually around 25 bp) have a much narrower window of signal to background and, therefore, are much more likely to yield background results than the full-length or so-called genomic probes (usually around 100 bp from much larger templates), given the much greater number of hydrogen bonds in the probe-target complex for the latter. For immunohistochemistry

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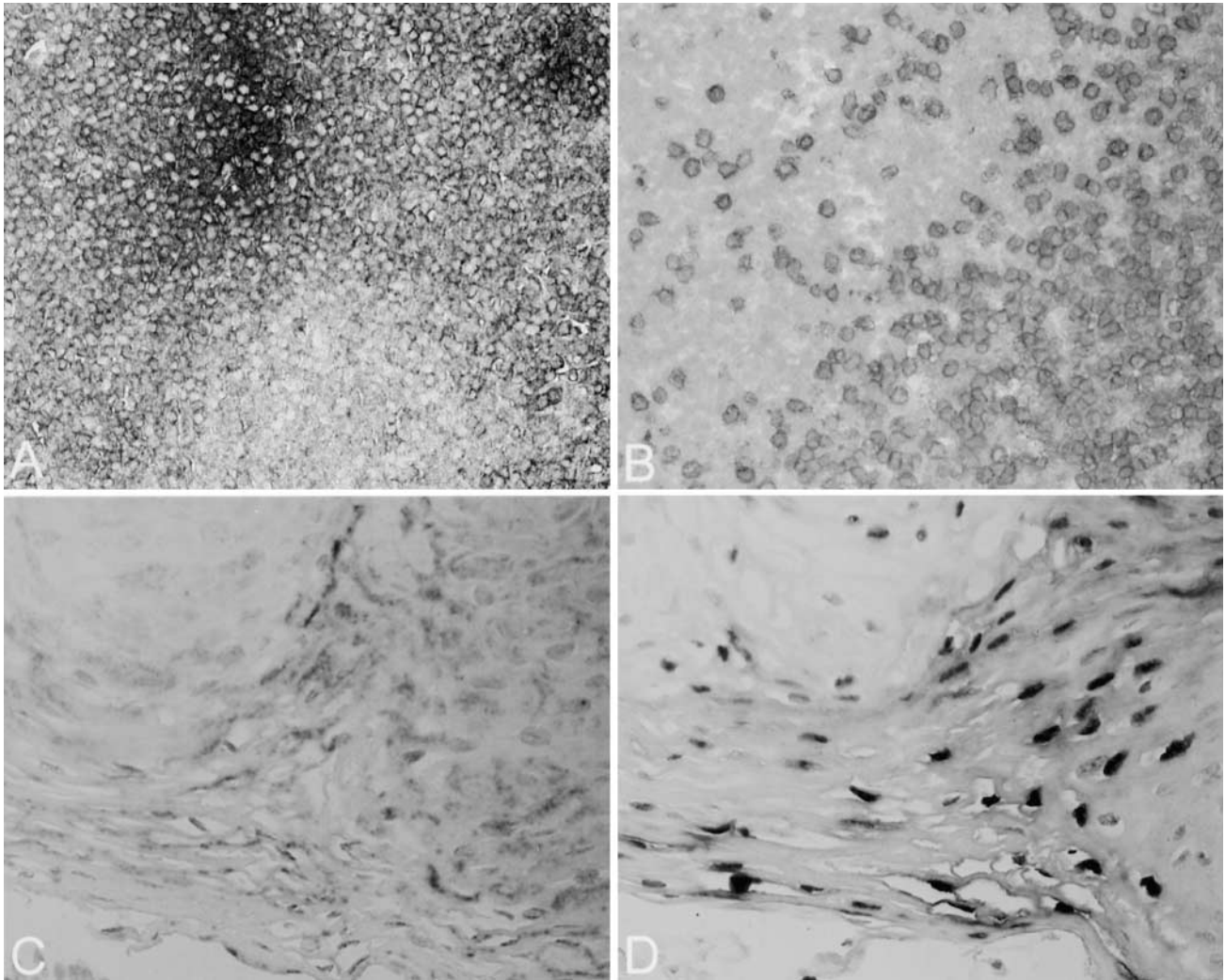


Figure 1 Background vs signal with IHC and ISH. (A) Strong background after IHC for CD3 in a lymph node where there was no protease pretreatment. Note how the background is lost with a concomitant strong, well-defined cytoplasmic signal in the T-cell-rich interfollicular zones after pretreatment in proteinase K (50 $\mu\text{g/ml}$) for 15 minutes (B). (C) Strong cytoplasmic background in a vulvar lesion after ISH using an HPV-6 probe at a concentration of 100 $\mu\text{g/ml}$. Note the loss of the background and the emergence of a strong nucleus-based signal after decreasing the probe concentration to 10 $\mu\text{g/ml}$ (D).

(IHC), suboptimal or protease over-digestion (or its equivalent, antigen retrieval), antibody concentration and, to a lesser extent, washes are three key variables that can easily produce background (Figure 1). The causes of background for PCR ISH are identical to those for standard ISH. The most likely cause of background with PCR in situ hybridization is due to the use of an oligoprobe (Nuovo et al. 1993,1994a; Nuovo 1997). Although it is true that primer oligomerization is a definite cause of background in solution-phase PCR, which necessitates the use of a probe (usually an oligoprobe) that does not include the primer sequences, this process does not appear to occur inside a cell during in situ amplification (Nuovo et al. 1993,1994a). It is tempting to speculate that this re-

lates to the high protein concentration of a cell, because certain proteins reduce primer oligomerization during the solution phase PCR (Nuovo et al. 1993; Nuovo 1997). Although the full explanation awaits further testing, it is important to appreciate that although mispriming and non-target DNA synthesis may cause background during PCR in situ amplification (requiring an appropriately stringent wash), primer oligomerization does not appear to play a role in background. Therefore, whenever possible one should use the larger full-length probes even if they include the primer sequences. This will enhance both the specificity and sensitivity of the assay.

RT in situ PCR is the only amplification technique that allows target-specific incorporation of the re-

porter nucleotide, thus eliminating the need for a hybridization step. Like any in situ molecular assay, it also is associated with the risk for a false-positive background. It can not be stressed enough that background with RT in situ PCR is highly correlated with the protease digestion time. Optimal protease digestion with RT in situ PCR is defined by a strong nonspecific signal in all cell types that is eliminated completely by overnight digestion with RNase-free DNase (Figure 2). If the tissue has not be adequately digested by protease, then the genomic DNA will not be completely accessible to the DNase, and nonspecific incorporation of the reporter nucleotide will occur in a process analogous to primer-independent DNA repair/nick translation. This leads to a false-positive result

confined to the nucleus that is easily recognized as such in analyzing for a cytoplasm-based signal. A simple way to detect this potential problem is to always run a control (DNase, no RT, or RT with irrelevant primers) in the tissue that one is testing. The presence of positive cells, or more specifically positive nuclei, indicates that the results are nonspecific (Figure 2). Furthermore, someone skilled in histopathology will also appreciate that the nonspecific signal will localize to the entire nucleus whereas the target-specific human RNA-based signal will be present in the cytoplasm. Of course, some viral RNAs are present in the nucleus, whereas others are compartmentalized in specific regions of the cytoplasm or—in cases—in the nucleus; for example, measles RNA can be detected in

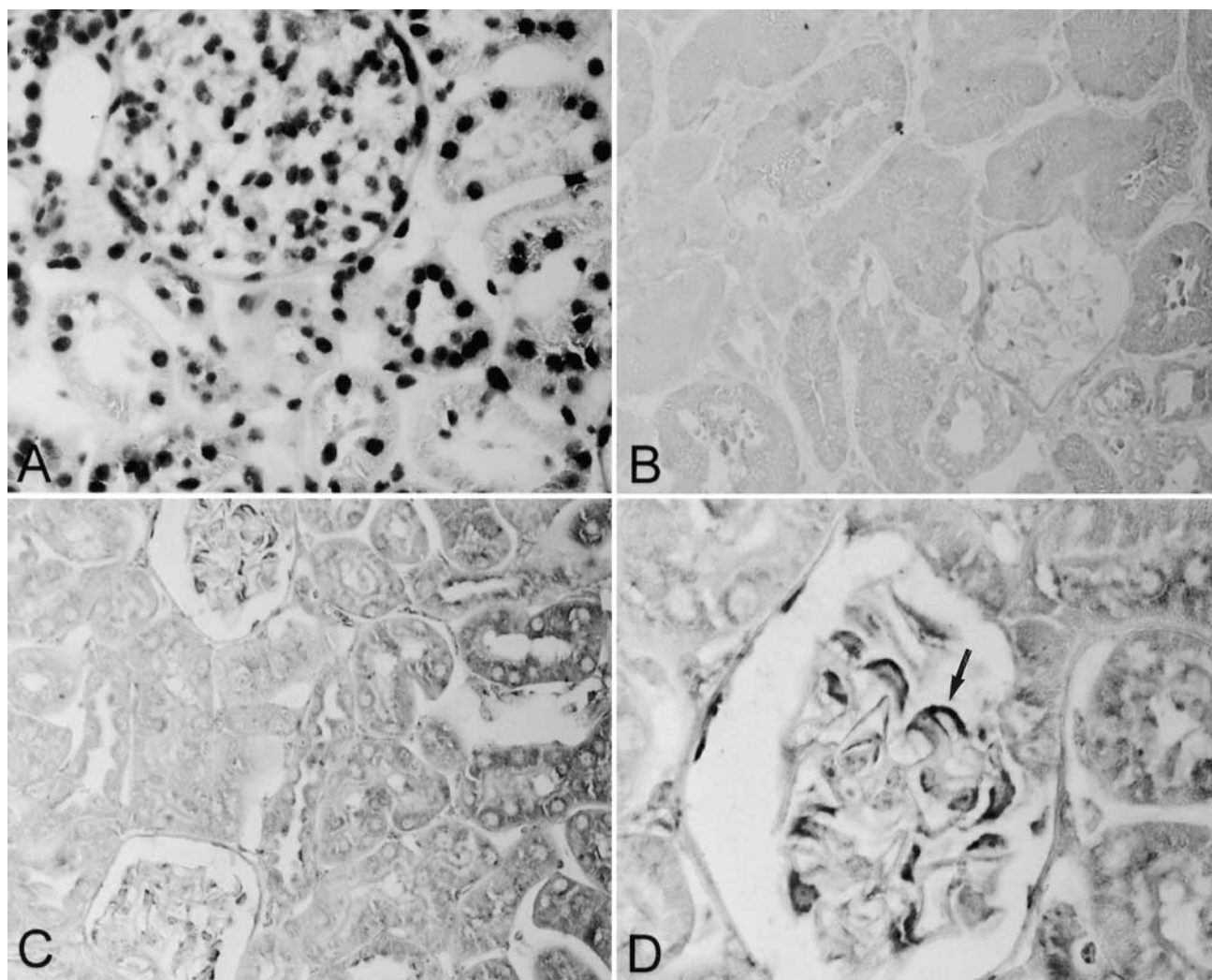


Figure 2 Background vs signal with RT in situ PCR. This renal biopsy was analyzed for mRNA by RT in situ PCR. After digestion for 10 min in pepsin (2 mg/ml) and DNase digestion, note the persistence of a nucleus-based signal when HPV-specific primers were employed (A; HPV cannot infect renal tissue). This nonspecific background was eliminated after 30 min of pepsin digestion and overnight DNase treatment (B). Under optimal protease conditions, the mRNA-based signal is evident in the cytoplasm of only the tubule cells and glomerular epithelial cells after RT in situ PCR with DNase digestion for α -adrenergic receptor message (C; higher magnification in D).

the cytoplasm or nucleus—in the latter case, it tends to have a peri-nucleolar distribution (Nuovo et al. 1992; Nuovo 1997).

Basic Methodology for Co-labeling Experiments

RNA Detection by RT In Situ PCR

Potential Sources of Background. To understand the RT in situ PCR methodology and the potential causes of background, it is important to be aware of the various pathways of DNA synthesis that may be operative inside an intact cell. Recall that the RT in situ PCR technique utilizes direct incorporation of the reporter nucleotide during PCR amplification. Clearly, the labeled nucleotide will be incorporated into any DNA molecule that is being synthesized, whether it is from target-specific cDNA or nonspecific DNA. Two sources of nonspecific DNA synthesis can be operative inside an intact cell: primer-independent DNA synthesis and mispriming (Nuovo et al. 1993,1994a). Nicks in the DNA induce the former pathway. The DNA polymerase can use any break in a phosphodiester bond to initiate DNA synthesis by displacing the native strand. The exonuclease activity of taq polymerase will also contribute to the primer-independent signal but is not prerequisite for such. Indeed, the ability of a DNA polymerase that lacks exonuclease activity to displace DNA at the site of any nick and thus to initiate DNA synthesis is the foundation of isothermal in situ amplification or, more specifically, strand displacement amplification (in situ SDA) (Nuovo 2000). Nicks in DNA can be generated from many sources, including exposure to dry heat, restriction endonucleases, sodium hydroxide or, importantly, paraffin embedding (Nuovo et al. 1993,1994a). It follows that any surgical or autopsy specimen that has been embedded in paraffin will demonstrate the primer-independent nonspecific DNA synthesis pathway. One can appreciate the robustness of this pathway by realizing that a signal can be generated from it within minutes of incubating the tissue with the polymerase. Mispriming generates DNA synthesis by extension of the primer that has annealed to non-target DNA. For primers that are 20–25 bp long (the usual size for oligoprimers), the massive amount of non-target DNA present in most samples increases the probability that some primers will anneal with non-target DNA long enough to support DNA synthesis (assuming that there is a bp match at the 3' end), even though the strength of hybridization of such complexes is weak owing to the low homology invariably present in this situation.

It is evident that there are two options for eliminating primer-independent DNA synthesis during in situ

PCR. One can either degrade the DNA using DNase digestion or never allow the DNA nicks to form. As indicated above, the latter is not possible with paraffin-embedded material. One can use frozen tissue samples that have been fixed in formalin for target-specific direct incorporation of the reporter nucleotide in DNA targets with in situ PCR, assuming that hot-start PCR is done to eliminate mispriming (see below), but such tissues are often not available. Degradation of the genomic DNA can be accomplished by a minimum of 7 hr of digestion in RNase-free DNase [10 U per tissue section; add 10 μ l of PCR Buffer II (Perkin Elmer; Norwalk, CT) to 80 μ l of RNase-free water to 10 μ l of RNase-free DNase (Boehringer Mannheim; Indianapolis, IN)], but only after adequate protease digestion. There is no more important concept to appreciate for those who wish to do RT in situ PCR. It is recommended to digest overnight with DNase to ensure that the entire amount of DNA inside the cell is sufficiently degraded to prevent any detectable DNA synthesis. Insufficient digestion with protease before DNase digestion will not permit DNase access to the genomic DNA, and therefore a nonspecific nucleus-based signal will persist with RT in situ PCR. The reason that protease digestion is so critical to successful RT in situ PCR is apparent when one realizes that formalin fixation induces protein–DNA crosslinks. The steric hindrance that persistent protease–DNA adducts causes to DNase digestion with suboptimal protease digestion will allow sufficient DNA nicks to persist that, in turn, will support DNA synthesis by taq polymerase. Indeed, the exonuclease activity of the taq polymerase and DNase may actually increase the amount of DNA available for primer-independent synthesis under suboptimal conditions of protease digestion and thus can explain the paradoxical increase in the strength of the primer-independent signal with in situ PCR and suboptimal protease digestion after DNase digestion (Nuovo et al. 1993,1994a).

Determination of optimal protease digestion time must be done for each tissue sample (or formalin-fixed cell suspension) that one wants to test for gene expression by RT in situ PCR. It is recommended to place three 4- μ m tissue sections on a given silane-coated slide to allow testing of three different protease digestion times on the one slide. Although each sample must be optimized for protease digestion before RT in situ PCR, there are some general principles that can help one better estimate probable optimal protease digestion times. Tissues very resistant to protease digestion include skin, skeletal muscle, and central nervous system (CNS) samples, which often require 60 min of digestion at 37C in high-concentration pepsin (2 mg/ml diluted in 0.2 N HCl) for optimal protease digestion. Tissues very sensitive to protease digestion in-

clude lymph nodes, tonsils, spleen and, to a lesser extent, liver and kidney, for which 20 min of pepsin digestion is often adequate. It is recommended to digest the three samples on a glass slide for 20, 40, and 60 min, respectively, followed by direct incorporation of the reporter nucleotide for 30 min at 55°C. The protease digestion time that gives the strongest signal in the greatest number of nuclei is the optimal digestion time. Using this protocol, one can make this determination in a few hours. Next, digest another slide from the same tissue sample for the optimal time and then subject at least one section on the slide to overnight DNase digestion. The complete elimination of the signal under these conditions confirms that one has the optimal protease digestion for that sample. If some signal persists after DNase digestion, re-do the experiment using longer digestion in pepsin. Over-digestion will be manifest by loss of the cell borders and cytoplasm and poor staining of the nuclei with the counterstain nuclear fast red (Nuovo 1997).

Choice of Reporter Nucleotide. The most commonly used reporter nucleotides for RT in situ PCR are digoxigenin and biotin. Digoxigenin offers the advantage of not being found in mammalian cells and therefore it eliminates one potential cause of background. Another advantage of digoxigenin is that excellent results can be obtained over a broad range of concentrations of the labeled nucleotide (usually digoxigenin-dUTP) to the unlabeled nucleotide (dTTP). Although the usual ratio is 1 labeled to 20 unlabeled, one can obtain good results with a ratio as high as 1:5 or as low as 1:80 (Nuovo 1997). Furthermore, there are many anti-digoxigenin conjugates that allow a wide choice of chromogens, including the most common alkaline phosphatase and peroxidase systems. This is especially important in co-labeling experiments for which one must use different reporter systems. Biotin will also give good results, although the concentration of labeled to unlabeled nucleotide [biotin-11-dUTP or biotin-7-dATP (Enzo Biochemistry; New York, NY)] should be 1:1. Another alternative is to use fluorescein-labeled nucleotides. Fluorescein and related tags (rhodamine and coumarin) may not give as reliably intense signals as those generated with biotin- or digoxigenin-labeled nucleotides if viewed directly using darkfield microscopy. An alternative is to use anti-fluorescein-alkaline phosphatase or peroxidase conjugates which do work well (Nuovo 1997).

Another alternative for in situ amplification of cDNA or DNA is to use tagged primers. This has the obvious advantage of eliminating any background from primer-independent DNA synthesis and therefore should theoretically allow target-specific direct incorporation for DNA targets. However, labeling primers that contain the maximal number of digoxige-

nin or biotin tags (three to five) may not permit a strong, reproducible signal (Nuovo 1997). This makes sense when one realizes that, with direct incorporation of a labeled nucleotide, on average one of every 20 nucleotides will be tagged. Thus, in an amplicon of 300 bp, 15 labels will be incorporated vs the three to five one can obtain with a labeled primer. Interestingly, fluorescein-tagged primers will yield a strong signal for RT in situ PCR and in situ PCR (for DNA targets, thus avoiding the hybridization step) if the "Sunrise" system is used (Nuovo et al. 1999). The Sunrise system employs a specific sequence on the target-specific primer that is homologous to the universal primer included in the reaction mixture. Hybridization of these DNA sequences permits separation of the quenching molecule from the fluorotag, which leads to the detectable signal (ONCOR; Gaithersburg, MD). This also offers the advantage of different colored fluorotags but is not further discussed here because of the relatively high cost of constructing these primers and the need for a fluorescent-based microscopy system; further information is provided in the reference (Nuovo et al. 1999).

The final choice to consider is radioactively labeled nucleotides, or actually ^3H , because the other radioisotopes (such as ^{32}P , ^{33}P , and ^{35}S) produce far too much background in terms of silver grains both over the tissue and away from the tissue (Nuovo et al. 1995; Nuovo 1997). ^3H also allows very sharp localization of the signal inside the cell. One problem using ^3H for standard (i.e., without any DNA or RNA amplification step) ISH is that it is a weak isotope and development times of 1 month or more may be needed. However, with direct incorporation of a ^3H -tagged nucleotide, one can generate strong signals after 1–2 days of exposure of the slide to the light-sensitive emulsion (NBT; Kodak, Rochester, NY). Its long half-life of almost 12 years is also advantageous. The main problem, of course, is the issue of working with and disposing of radioactive materials. For this reason, it is recommended to restrict the use of ^3H to co-labeling experiments. It is important to appreciate that the ratio of hot to cold nucleotide is critical and should be kept at 5:1 (Nuovo 1997).

It has long been agreed that biotin- or digoxigenin-tagged probes are as sensitive as radioactively labeled probes for ISH and PCR ISH (Crum et al. 1988; Walboomers et al. 1988), and therefore it is recommended that one use the former unless co-labeling experiments are planned.

Reaction Solution and Cycling Parameters. The reaction solution for RT in situ PCR is very similar to that for solution-phase RT PCR, with the exception that one includes bovine serum albumin (BSA), to minimize adsorption of the rTth polymerase to the

glass slide: 10 μ l 5 \times rTth buffer + 1.6 μ l dATP, dCTP, dGTP, dTTP mixture (final concentration 200 μ M) + 0.6 μ l digoxigenin dUTP (stock concentration 1 mM, final concentration 10 μ M) + 1.6 μ l 2% BSA + 3 μ l primer 1 and 2 (final concentration 20 μ M) + 2 μ l rTth DNA polymerase (Perkin Elmer) + 12.4 μ l Mn-acetate (or MnCl) (final concentration 2.5 μ M) + 14 μ l RNase-free water.

After determining the optimal protease digestion time for the particular sample, as described above, pretreat two of the three sections on the glass slide with 10 U per section of RNase-free DNase. Next, prepare the two vials containing the rTth solution (or prepare 50 μ l and divide into 20- μ l and 30- μ l aliquots). To one vial/aliquot add the target-specific primer set. To the other vial/aliquot add irrelevant primers [virus-specific primers are a good choice; for example, human papillomavirus (HPV) cannot infect most tissues]. Place the solution over the section and prevent evaporation either with a polypropylene coverslip that is anchored with nailpolish and overlaid with mineral oil, the Amplicover system of Perkin Elmer, or the SelfSeal system of MJ Research Laboratory. Incubate for 30 min at 60C, followed by denaturation at 94C for 3 min, then do 20 cycles at 60C for 1.5 min and 94C for 45 sec. It is very important to wash the slides at 60C in 0.1 \times SSC/2% BSA for 10 min to remove any labeled primer dimers that may be in the amplifying solution and weakly binding to cellular proteins and nucleic acids. For digoxigenin- or biotin-tagged systems, use the appropriate alkaline phosphatase-labeled conjugate (37C for 30 min), followed by development in the chromogen nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP). Monitor the progression of the signal under a microscope; often 5–15 min will be sufficient. Counterstain for 5 min in nuclear fast red, dehydrate in 100% ethanol, then coverslip with permount out of xylene. A successful experiment is defined by a strong nucleus-based signal in all cell types in the non-DNase-digested tissue and lack of any signal in the DNase-digested tissue incubated with the irrelevant primers. If both criteria are not present, the experiment must be redone, altering the protease digestion time.

DNA Detection by PCR ISH (or ISH)

ISH and PCR ISH are certainly less fastidious than RT in situ PCR in the sense that much less emphasis must be placed on optimizing the variables needed for a successful experiment. Although one needs to determine for any given sample the optimal protease digestion with RT in situ PCR, for ISH and PCR ISH 30-min digestion at 37C using 2 mg/ml of pepsin (diluted in 0.2 N HCl) will be adequate for over 90% of samples (Nuovo 1997).

A simple protocol is recommended for ISH using paraffin-embedded, formalin-fixed tissues. Removal of the paraffin is accomplished by 3–5 min in fresh xylene, followed by 3–5 min in fresh 100% ethanol. After air-drying the slide, the tissue is digested for 30 min in pepsin (2 mg/ml) that is washed off using a water rinse for 1–2 min, followed by a dip in 100% ethanol to assist in air-drying the slide. A small amount of probe cocktail (5–10 μ l) is placed over each tissue section and covered by a polypropylene coverslip cut to size. This way, one can test three or four probes per glass slide. The probe cocktail contains the following: 50 μ l deionized formamide (use 10 μ l for oligoprobes and add 40 μ l water) + 30 μ l 25% dextran sulfate + 10 μ l 20 \times SSC + 10 μ l of the probe (stock solution of 5–10 μ g/ml).

Co-denature the probe and tissue DNA by heating at 95C for 5 min, and hybridize for 2–15 hr at 37C in a humidity chamber or the probe cocktail will dry out. This should be followed by the standard protocol: a high-stringency wash (0.2 \times SSC in 2% BSA for 10 min at 60C for full-length probes), 20–30 min in the alkaline phosphatase conjugate (typically streptavidin or anti-digoxigenin), then 30–60 min in the chromogen. Nuclear fast red counterstaining for 5 min provides a light but distinct pink color in the negative cells if NBT/BCIP is used, allowing good morphological detail, and permits better detail when the data are photographed. This system allows coverslipping through xylene into permount, where the color-based signal will remain intact for many years.

The key concept for PCR ISH is the hot-start maneuver. By not allowing the taq polymerase to become active until a temperature threshold is reached (usually 55C), one prevents mispriming. The end result is an increase in the sensitivity and specificity of both solution-phase PCR and PCR ISH (Nuovo et al. 1991; Chou et al. 1992). Indeed, detection of one copy by PCR or PCR ISH requires the hot-start maneuver. There are several ways to do hot-start PCR. The simplest way is to withhold the taq polymerase until the reaction temperature reaches 55C. Alternatively, one can use chemicals that interfere with taq polymerase function at lower temperatures but are themselves inactivated at temperatures above 55C. Examples of the latter include antibodies specific for taq polymerase (AmpliGold; Perkin Elmer) and the enzyme uracil N-glycosylase which degrades any DNA containing dUTP (which must of course be included in the reaction mixture) (Nuovo 1997). Another option is to use proteins that can bind the primers and inhibit their interaction with non-target DNA at temperatures below 55C. One example is single-stranded binding proteins (Nuovo et al. 1991). Of these choices for PCR ISH, either withholding the taq polymerase from the reaction mixture until 55C is reached or using AmpliGold or a

similar product are the most commonly used procedures. The reaction mixture should contain: 5 μ l GeneAmp buffer + 9 μ l MgCl₂ (25 mM stock) + 8 μ l dNTPs (2.5 mM stock) + 2 μ l each primer 1 and primer 2 (each 20 μ M stock) + 2 μ l 2% BSA + 31 μ l sterile water + 1 μ l of taq polymerase. Note the relatively high concentration of magnesium relative to solution-phase PCR and the use of BSA, which increases the effective concentration of the taq polymerase by inhibiting its adsorption to the glass (Nuovo 1997).

The approach to solving problems of background with ISH is similar to that for PCR ISH if one uses, as is recommended, full-length (and not oligo) probes. Specifically, the important variables to consider when background is evident are probe concentration, hybridization time, and the post-hybridization wash conditions. Probe concentration may be a problem when concentrations greater than 10 μ g/ml are used. If background is evident, a first step to consider is reducing the concentration of the probe by 1:10 (Figure 1). It should be stressed that probe concentrations greater than 10 μ g/ml will often not give background but rather yield a strong, clear signal. It is recommended to test each new probe initially over a wide range of concentrations with tissues that do not contain the target to determine the highest concentration of probe that will not be associated with background. Post-hybridization wash stringency is another common cause of background; washes below 52C increase the risk for background. Therefore, a wash that is only a few degrees below the melting temperature for probe-target complexes of 100 bp (the typical size with ISH) is recommended to minimize any chance of background. This would be between 60 and 65C if one uses 0.1 \times SSC without, of course, any formamide. Hybridization time is usually not a problem with ISH, although it certainly can be if one uses oligoprobes. One should limit the hybridization with an oligoprobe to 2 hr and, as described in detail elsewhere, reduce the post-hybridization wash to around 50C with a tenfold higher concentration of salt (Nuovo 1997). Given the narrow window between signal and background with oligoprobes, one should expect to do much trial-and-error testing to determine the correct post-hybridization wash and probe concentration when an oligoprobe is used.

Protein Detection by Immunohistochemistry

The detection of proteins by IHC using paraffin-embedded, formalin-fixed tissue is something that every diagnostic pathology laboratory is familiar with. Typical protocols include incubation with a primary antibody for 1–2 hr, followed by treatment with a secondary biotin-labeled antibody directed against the animal from which the primary antibody was obtained. This allows subsequent treatment with a streptavidin conjugate (usu-

ally linked with peroxidase) followed by exposure to the chromogen, in this case DAB. A rapid counterstain in hematoxylin and mounting with permount complete the process. The topic of signal and background with IHC has received a great deal of attention and reminds us that this is by no means a problem unique to RT *in situ* PCR. Most laboratories, mine included, spend much time trying to resolve background issues with IHC. This is simply because each antibody-antigen complex must be separately tested and such complexes will have fewer hydrogen bonds than a DNA-cDNA target complex, making background often more difficult to eradicate.

Most antibodies are purchased commercially and of course include directions for optimal concentration and pretreatment (antigen retrieval, protease digestion, or no pretreatment). When a new antibody is tested it is recommended to take a tissue known to contain the antigen and routinely test no pretreatment, weak protease digestion (proteinase K 50 μ g/ml for 5 min), strong protease digestion (proteinase K 250 μ g/ml for 15 min), and antigen retrieval. By placing multiple sections per slide and using polypropylene coverslips, one can do these tests with only two or three slides. In most cases, one of these reaction conditions clearly gives the strongest signal-to-background ratio (Figure 1). If background is still a problem, re-do the experiments diluting the antibody 1:10.

Co-detection of a Nucleic Acid and a Protein

When co-labeling experiments to detect a protein and nucleic acid in a paraffin-embedded, formalin-fixed tissue section are planned, one must first determine if the antigen will show an adequate signal after the protease digestion required for *in situ* amplification. An advantage of pepsin (compared to proteinase K) digestion is that pepsin is much more efficient at preserving tissue morphology. However, low concentrations of proteinase K (10–100 μ g/ml) may be satisfactory. A simple way to determine the effect of protease digestion on the detection of the antigen in formalin-fixed material is to use one silane-coated glass slide that has three tissue sections on it. The first section gets no protease, while the second and third sections are digested in pepsin (2 mg/ml) for 30 and 60 min, respectively. This is followed by standard IHC for the antigen of interest. A good signal-to-background ratio at 30 or 60 min will be a good indication that one can do co-labeling of the antigen with a specific DNA or RNA sequence. Obviously, a poor signal would suggest that such co-labeling experiments will not be possible. An alternative, especially with cell preparations, is to use another fixative, such as ethanol or acetone, which might permit antigen detection with much weaker protease digestion times. Although this

may work well with cytological preparations, it tends not to work as well with tissue sections (Nuovo 1997). Fortunately, many antigens will show a strong signal after pepsin digestion for 30–60 min. A short and by no means complete list includes most of the intermediate filaments (cytokeratins, vimentin, GFAP, actin) and many of the lymphoid markers (CD3, CD68, CD45). However, other lymphoid markers, such as CD4 and CD8, are likely to show background with poor signal after in situ amplification (Nuovo et al. 1994b, 1996; Nuovo and Alfieri 1996).

If one has determined that the antigen of interest will perform well after protease digestion, it is recommended to do the in situ amplification first, followed by the IHC. The protocol one follows is as outlined above for PCR ISH or RT in situ PCR, with one exception. Do not counterstain the slide but rather transfer the slides from water to either PBS or TBS and proceed with the immunohistochemical detection. After both steps are done, it is recommended not to counterstain the slides.

The key decision to make when co-labeling experiments are performed is the choice of chromogen. For ISH, PCR ISH, or RT in situ PCR, it is recommended to use digoxigenin with either NBT/BCIP (blue) or fast red as the chromogen. The blue or red color offers good contrast with the brown of DAB. This is especially true if the signals for the nucleic acid and protein are located in different compartments. Figure 3 shows a representative example in which the blue nucleus-based in situ PCR signal of HPV is clearly distinct from the brown cytoplasmic based signal of cytokeratin. Figure 3 also shows an example in which both signals are present in the cytoplasm (T-cell receptor beta mRNA after RT in situ PCR and CD3 after IHC). Although it is possible to determine which cells co-express CD3 and the specific T-cell receptor beta mRNA of interest, in such instances it may be advantageous to use tritium.

Many different laboratories have reported protocols that offer co-localization of a DNA or RNA target by ISH or, less commonly, in situ PCR with IHC (Matsuki et al. 1992; Gosztonyi et al. 1994; Kriegsmann et al. 1994; Nuovo et al. 1994b; Matsuno et al. 1996; Nuovo and Alfieri 1996; Rimsza et al. 1996; Nuovo 1997; Wesselingh et al. 1997; Nichol et al. 1999; Ozawa et al. 1999). Typically, these protocols employ two chromogens, often NBT/BCIP, DAB, fast red, and/or new fuschin. In addition, most recommend doing the ISH analysis first, followed by IHC. Finally, Matsuno et al. (1996) have reported on a protocol allowing co-localization of an mRNA and a protein after electron microscopy-based ISH and IHC.

Tritium allows the most distinct co-localization with a chromogen-based signal, especially when both the protein and nucleic acid localize to the same sub-

cellular compartment (Nuovo and Alfieri 1996) (Figure 3). Recall that with tritium for RT in situ PCR it is very important to use the correct ratio of cold to hot nucleotide (1:5) and to expose the slide to the light-sensitive emulsion for no more than a few days. An important technical tip for using tritium (either labeled probes or direct incorporation of the labeled nucleotide) is that one must thoroughly wash the slide in 100% ethanol (5 min) and xylene (30 min with shaking) to completely remove the emulsion from the slide, otherwise one will not be able to detect the chromogen-based signal.

Co-detection of Two Nucleic Acid Targets

Clearly, one can co-localize either two DNA targets or two RNA targets if in situ amplification is used (and not a RNA and DNA target), due to the obligatory DNase digestion one must employ for detecting RNA targets by direct incorporation of the labeled nucleotide and RT in situ PCR. In such cases, one can do RT in situ PCR using tritium or biotin with one primer-specific pair, remove the mineral oil, and then do another RT in situ PCR reaction on the same slide using digoxigenin as the reporter system with the other cDNA primer-specific set. After the second reaction is finished, one must either detect the tritium (with an extensive post-development wash in xylene as indicated above) or biotin followed by detection of the digoxigenin. When biotin and digoxigenin are used, one can use alkaline phosphatase/NBT and BCIP for biotin followed by detection of the digoxigenin using anti-digoxigenin conjugated to peroxidase, with DAB as the chromogen (Figure 3).

Application to HIV-1 Pathogenesis

There are, of course, many instances in which it would be very useful to know if a given cell contains a specific DNA or RNA sequence and the antigen to mark its phenotype. It is beyond the scope of this review to discuss many examples of the utility of co-labeling. HIV-1 infection is used as one example of the useful information one can obtain by detecting both a nucleic acid sequence and a specific antigen in one paraffin-embedded, formalin-fixed tissue section.

Co-labeling experiments using RT in situ PCR have helped a great deal in elucidating certain critical aspects of HIV-1 pathogenesis. For example, it can be demonstrated that lymphocytes infected with HIV-1 co-label with CD4 and, more importantly, include on average 30% of such cells in the lymph nodes even while the patient is asymptomatic (Bagasra et al. 1992; Embretson et al. 1993; Nuovo et al. 1994c). This information, available only after in situ PCR amplification of the provirus, was critical evidence in the documentation that HIV-1 infection is the direct cause

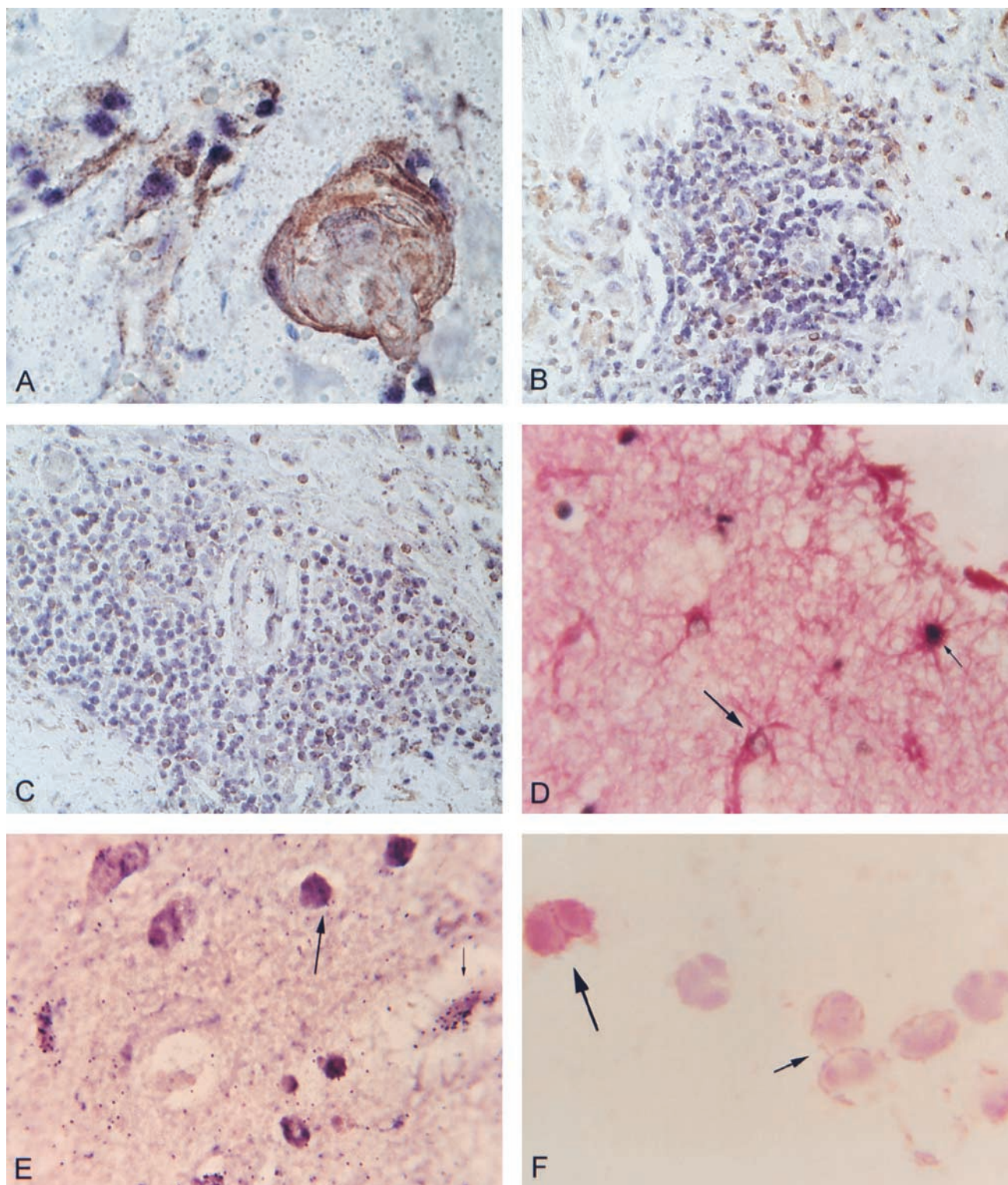


Figure 3 Co-labeling using different chromogens and tritium. (A) A nest of cells in the pelvic wall region from a woman with cervical cancer. All cells are, as expected, cytokeratin-positive (brown cytoplasm) but only about half of the cells express the viral oncotranscript E6 (blue, nucleus-based). (B,C) Co-labeling for CD3 (brown) and two different T-cell receptor beta (TCRB) mRNAs (blue) in this inflammatory skin process, i.e., there is a polyclonal pattern of expression. (D) Co-localization of HIV-1 RNA (blue, nuclear) and GFAP (astrocytes; red, cytoplasmic), using fast red as the chromogen for the IHC. Small arrow depicts an HIV-1-infected astrocyte and large arrow an HIV-1-negative astrocyte. (E) Co-localization of HIV-1 RNA (blue nucleus-based signal, large arrow) and iNOS mRNA (tritium-based signal, small arrow). The cytokine is expressed in HIV-1-negative cells directly adjacent to the infected cells. (F) The use of fast red (HIV-1 DNA after PCR ISH) and DAB (CD4, brown rim) as for co-labeling; the small arrow depicts non-infected CD4 lymphocytes and large arrow two HIV-infected CD4 cells.

of AIDS, a well-accepted belief that not too long ago was much disputed. This has become the basis for the use of anti-viral cocktails to treat HIV-1 infection, which have helped tremendously in extending the life of infected people. It also has been documented that the lymph node is the primary reservoir of HIV-1 infection and that the CD4 and CD21 (follicular dendritic cells) are the two primary targets. At end-stage AIDS, most of the remaining CD4 cells are actively infected by the virus and the CD21 cells are no longer detectable, consistent with the slow and inexorable destruction by the virus (Nuovo et al. 1994c).

Another area in which co-labeling experiments have provided useful information is in AIDS-related dementia, one of the most devastating of the disease complexes that mark the acquired immunodeficiency disorder. Many theories have tried to explain the devastating CNS symptoms found in AIDS dementia including, but not limited to, infection by HIV-1, infection by other viruses, upregulation of cytokines, crossreactivity between HIV-1 antigens and CNS-specific epitopes, and direct neuron toxicity of virus-related proteins (Nuovo et al. 1994b; Nuovo and Alfieri 1996; Dubrovsky et al. 1995; Schmidtmayerova et al. 1996,1998). One reason for such a plethora of theories is that it is difficult to detect in situ either HIV-1 antigens, DNA (provirus), or RNA. PCR ISH is required to detect the single proviral copy typical of latent (or in many cases) productive infection. PCR ISH has demonstrated that HIV-1 DNA is routinely found in perivascular spindle-shaped to round cells in the CNS in people with no evidence of AIDS dementia. These cells co-label with RCA-1 and CD68 and therefore are microglial cells/macrophages. Viral RNA is rarely present in such cells, and hence the infection is latent. Furthermore, the mRNA for a variety of cytokines including tumor necrosis factor alpha (TNF α), inducible nitric oxide synthetase (iNOS), and macrophage inhibitory factor alpha or beta (MIPA, MIPB), are detectable in, at most, a few cells. In AIDS dementia, there is a massive increase in the number of cells that contain HIV-1 DNA. Co-labeling experiments show that these cells co-label with RCA-1, GFAP, or neuron-specific enolase, and therefore they represent microglial cells, astrocytes, and neurons, respectively (Figure 3). Most of the infected cells also contain spliced viral transcripts indicative of productive infection. Interestingly, there is a large increase in the area that contains HIV-1-infected cells (typically at the junction of the gray and white matter in the area of the cerebral cortex) in the number of cells expressing either TNF α , iNOS, MIPA, or MIPB. An obvious and important question is, are the HIV-1-infected cells expressing the cytokines or are the neighboring non-infected cells the source? Co-labeling experiments have shown that it is the HIV-1-negative

cells directly adjacent to the HIV-1-infected cells that are responsible for cytokine production, suggesting that the productive viral infection may, in part, be responsible for the host response of cytokine overproduction (Figure 3). Because cytokine overexpression can be directly injurious to neurons, the data derived from in situ amplification and co-labeling experiments suggest a multifactorial pathogenesis involving direct and massive productive infection by the virus and an associated "over-robust" host cytokine response (Nuovo et al. 1994b; Nuovo and Alfieri 1996).

It is important to stress that, whereas for HIV-1 DNA detection one must employ PCR ISH for provirus detection with co-labeling experiments to maximize sensitivity, there are other situations in which the target number is already high and ISH would offer adequate sensitivity. An example would be the detection of a productive infection by any DNA virus, such as HPV, cytomegalovirus, *Herpes simplex* virus, and adenovirus, to give a few examples. Infections by these viruses are associated with hundreds to thousands of copies of viral DNA per cell, and therefore standard ISH offers excellent sensitivity and specificity for co-labeling experiments. However, for latent virus infection (such as HIV-1), point mutations, and many viral RNAs, standard ISH does not offer high enough sensitivity for routine detection directly or, of course, for co-labeling experiments. In situ amplification must be employed under these conditions.

Conclusion

In summary, if one focuses on the potential causes of background with in situ hybridization, PCR in situ hybridization, RT in situ PCR, and immunohistochemistry, one can often obtain good results in co-localizing a specific protein and nucleic acid molecule or two nucleic acid molecules in paraffin-embedded, formalin-fixed tissues. For immunohistochemistry, it is important to determine the effect of protease digestion (30–60 min with 2 mg/ml of pepsin) on the signal-to-background ratio. For RT in situ PCR, the key variable is optimal protease digestion time, defined as an intense primer-specific signal eliminated completely by overnight DNase digestion. For in situ hybridization and PCR in situ hybridization, probe concentration and the stringency of the post-hybridization wash are the key variables. It is recommended to do the DNA or RNA detection first (by in situ hybridization for high-copy targets and in situ amplification for low-copy targets), followed by detection of the protein by immunohistochemistry. In most instances, one can do co-labeling experiments using two different colorimetric-based detection systems; NBT/BCIP, new fuchsin, or fast red with alkaline phosphatase and DAB with peroxidase offers good contrast. Tritium may be needed

for nucleic acid detection when the two sequences of interest localize to the same compartment. Co-labeling experiments provide additional and powerful information in areas such as the pathogenesis of viral infections and oncogenesis.

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