

# **Tyramide Signal Amplification Strategies for Fluorescence Labeling**

**Kevin A. Roth, MD, PhD**

Department of Pathology, University of Alabama at Birmingham  
Birmingham, Alabama

---

## Introduction

The sensitivity of immunohistochemical (IHC) fluorescence labeling is ultimately determined by the properties of the fluorophore used for detection and the amount of fluorophore present at the sites of antibody binding. IHC antigen detection limits can therefore be lowered using enzymatic amplification procedures that deposit additional fluorophore molecules at the sites of antibody binding. Tyramide signal amplification (TSA) has proven to be a particularly versatile and powerful enzyme amplification technique for improved IHC detection. TSA can be utilized in either single or multilabel IHC applications and can be coupled with quantum dots for dramatic fluorescence signal photostability and accurate quantitation of fluorescence signal intensity. This chapter will briefly review both TSA and quantum dot IHC techniques, followed by examples of their use in my laboratory, and finally, provide useful troubleshooting strategies and tips for improving TSA IHC detection.

## Tyramide Signal Amplification

Traditional enzymatic IHC detection methods have utilized the ability of horseradish peroxidase (HRP) or alkaline phosphatase (AP) to convert a chromogenic substrate into a colored reaction product that precipitates at the site of enzymatic activity (Roth and Baskin, 2005; Roth and Perry, 2005). Alternatively, a few soluble HRP and AP substrates that are converted into insoluble fluorescent reaction products have been described for IHC detection but have not achieved wide use. The sensitivity of HRP or AP detection can be improved upon by using “layering” techniques such as peroxidase-anti-peroxidase (PAP) or avidin-biotin complexes (ABC) that increase the number of enzyme molecules associated with the primary antibody. Similarly, polymer systems that increase the number of enzyme molecules directly linked to detection antibodies can improve IHC sensitivity.

For many IHC applications, these traditional enzymatic amplification procedures are sufficient for achieving adequate antigen detection. However, several factors limit the sensitivity and utility of these procedures:

- A threshold amount of reaction product must be generated in order for precipitation to occur;
- Diffusion of the reaction product may be substantial;
- The precipitated reaction product may not be permanent (i.e., it may exhibit some solubility over time in mounting medium);

- The precipitates may be coarse or very large (which prohibits precise antigen localization); and
- Multilabeling options may be limited.

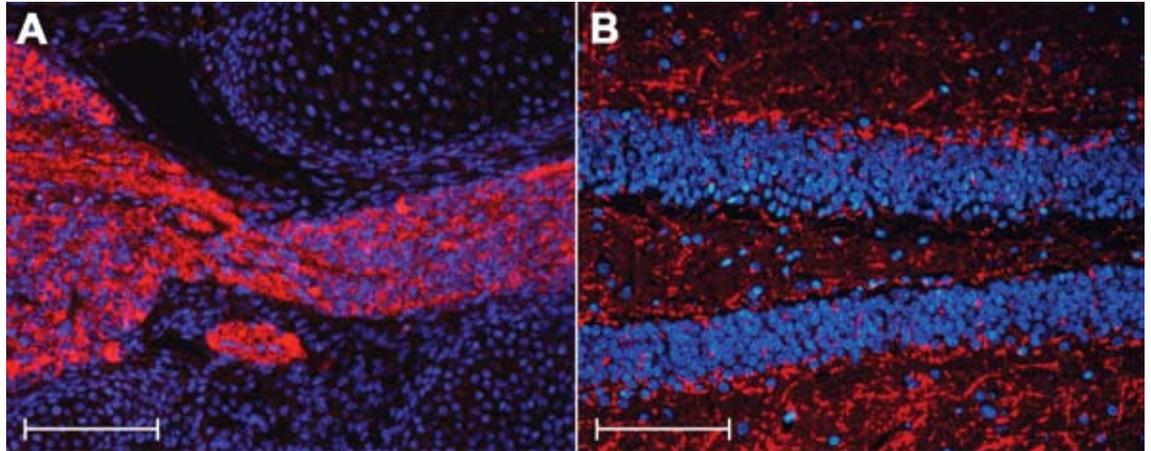
For these reasons, the development of tyramide signal amplification (TSA, Perkin-Elmer, Waltham, MA) techniques for IHC detection represented a major advance in the field.

TSA is based on the ability of HRP, in the presence of low concentrations of  $H_2O_2$ , to convert labeled tyramine-containing substrate into an oxidized, highly reactive free radical that can covalently bind to tyrosine residues at or near the HRP (Bobrow et al., 1992; Adams, 1992; Shindler and Roth, 1996). To achieve maximal IHC detection, tyramine is pre-labeled with a fluorophore, which is directly visualizable upon its deposition, or a hapten, which is then detected in subsequent steps with a hapten-specific reagent linked to a fluorophore or an enzyme molecule that can be used to deposit chromogen (Fig. 1). Fluorescent and hapten-labeled tyramides are available commercially from several sources (Perkin Elmer; Invitrogen, Carlsbad, CA; Dako, Carpinteria, CA).

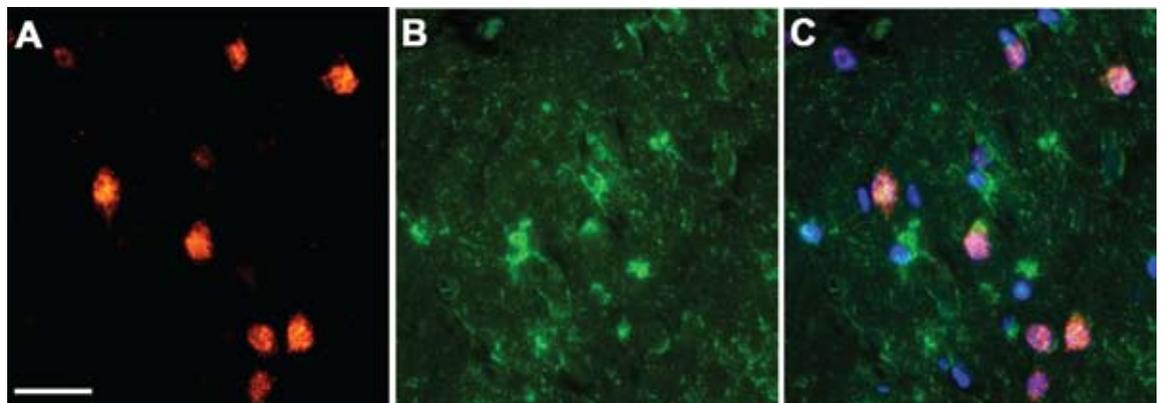
TSA has been successfully employed for both IHC detection (Fig. 2) and *in situ* hybridization (ISH) (Fig. 3) procedures (Zaidi et al., 2000; Roth, 2002; Ness et al., 2003; Akhtar et al., 2007). Because TSA results in the covalent linking of labeled tyramide to the solid phase (i.e., tissue section or cells), it differs fundamentally from other enzyme reaction products, which deposit owing to their precipitation. TSA is also much more sensitive than conventional fluorescence IHC detection, which utilizes secondary antibodies labeled with a fluorophore. In contrast, TSA results in the deposition of many more fluorescent molecules than can be linked to secondary antibodies.

Further improvements in TSA-based IHC detection have been achieved by optimizing the TSA reaction buffer (Roth and Baskin, 2005). The commercially available TSA Plus system (Perkin Elmer) uses greater concentrations of inorganic salts and a catalyst to increase tyramide deposition. When using TSA Plus, it is important to be aware of potential artifacts that may be associated with this procedure. Because the optimized reaction buffer results in the generation of more reactive tyramide intermediates, there is increased tyramide dimer formation in solution. We have found that tyramide dimers have an affinity for elastin and collagen, and this can lead to primary antibody-independent labeling of connective tissue or elastin containing blood vessels. To

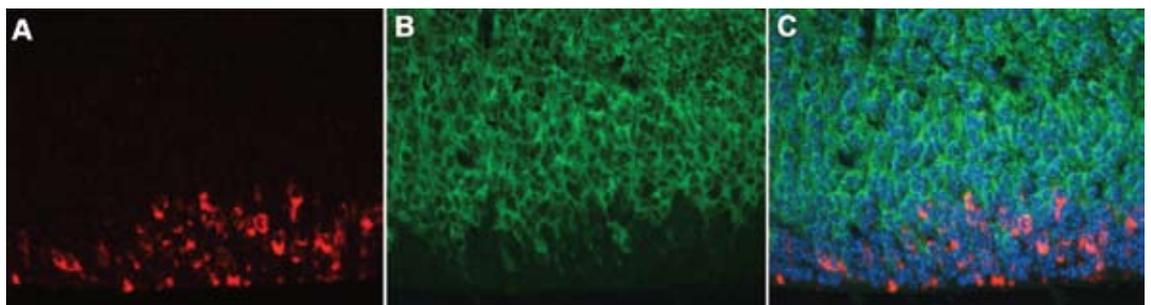
## NOTES



**Figure 1. TSA-IHC detection in nervous system tissue.** *A*,  $\beta$ -tubulin immunoreactivity is observed in gestational day 14 dorsal root ganglia of the mouse using cyanine 3-tyramide (red). *B*, Neurofilament immunoreactive neuronal processes are seen in the adult mouse hippocampus. Nuclei are labeled in both sections by Hoechst 33,258 (blue). Scale bars equal 50 microns.



**Figure 2. Dual-label TSA IHC detection.** *A*, Neurons in the adult mouse brain were detected with mouse anti-NeuN monoclonal antibodies and cyanine 3-tyramide. *B*, Glial fibrillary acidic protein (GFAP) immunoreactive cells were detected with rabbit polyclonal antiserum and fluorescein-tyramide. *C*, Merged image of NeuN, GFAP, and Hoechst 33,258 stained nuclei. Scale bar equals 20 microns.



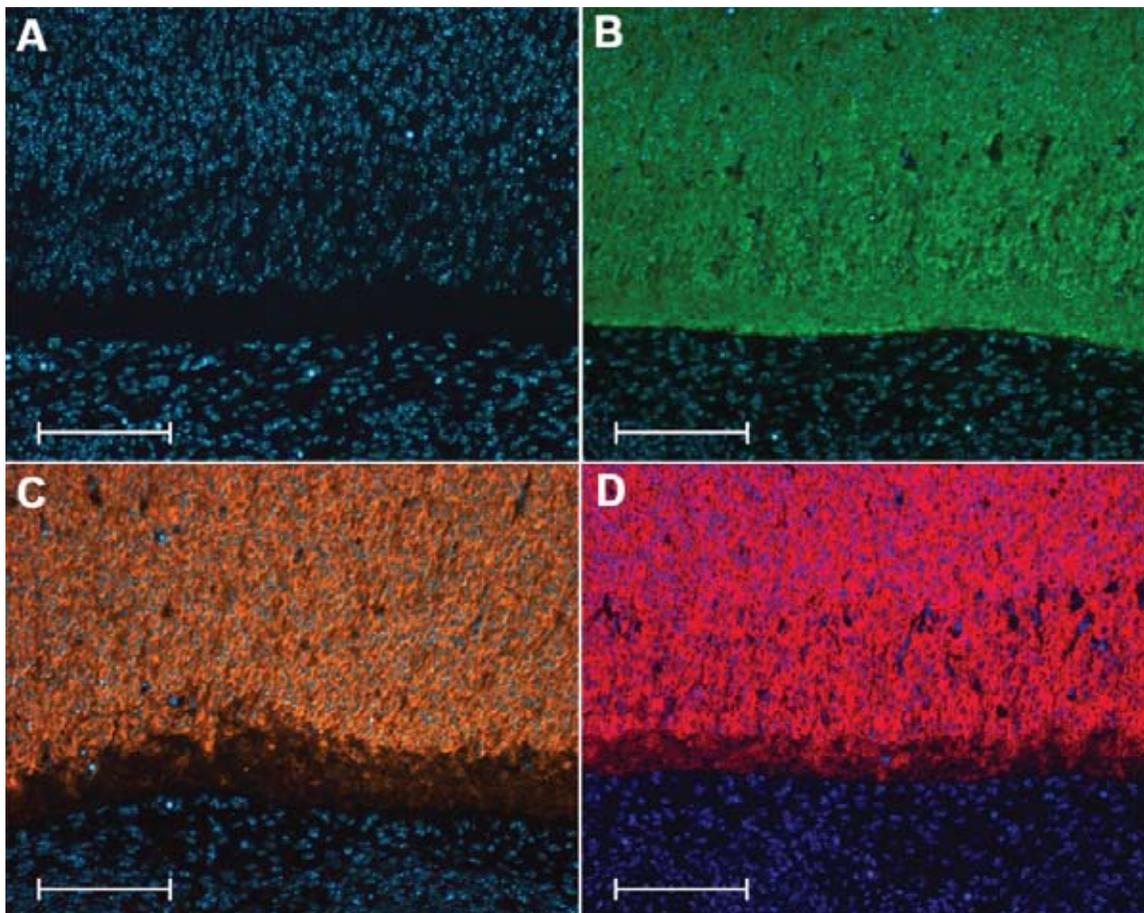
**Figure 3. Dual TSA ISH and IHC detection.** *A*, A section of mouse embryonic brain shows expression of histone H4 mRNA in proliferating neural precursor cells in the ventricular zone detected by digoxigenin-labeled cRNA probes and TSA Plus cyanine 3-tyramide. *B*, Microtubule-associated protein 2 (MAP2) immunoreactive neurons were identified using a monoclonal antibody and TSA Plus fluorescein-tyramide. *C*, Merged image of H4 mRNA containing neural precursor cells, MAP2 immunoreactive neurons, and Hoechst 33,258 stained nuclei.

minimize tyramide dimer formation, the concentrations of the HRP-labeled secondary antibody and the tyramide solution need to be optimized (Roth and Baskin, 2005).

### Quantum Dots

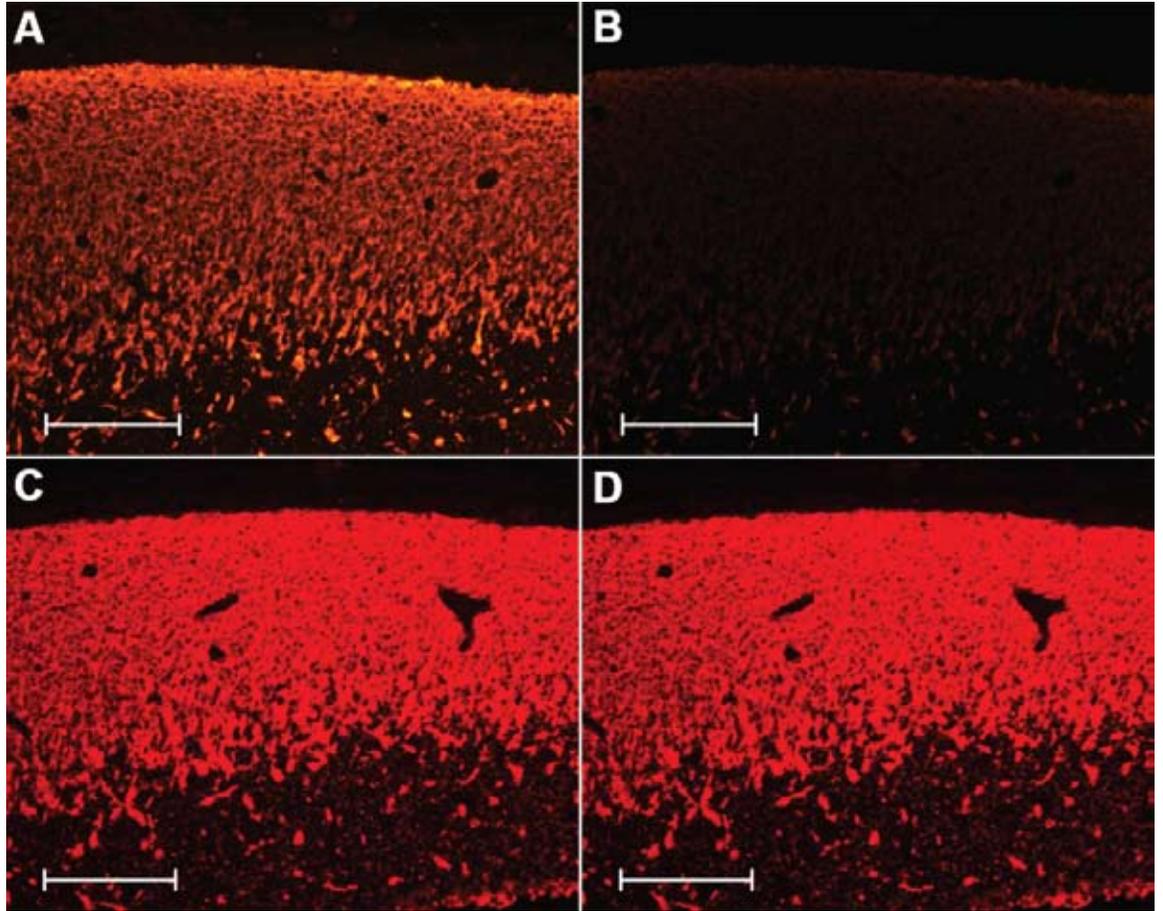
Quantum dots are nanocrystals consisting of a semiconductor core of cadmium selenide, coated with a shell of zinc sulfide, and a polymer layer that enhances water solubility. The polymer layer permits conjugation of the quantum dot to other molecules such as streptavidin or immunoglobulin (Wu et al., 2003). Quantum dots have an apparently large Stokes shift, narrow emission spectra, and remarkable photostability (Watson et al., 2003). Excitation is achieved over a very broad spectrum, and peak emission is determined by the size of the semiconductor core (Fig. 4).

Although quantum dots have many beneficial properties that potentially increase their utility over that of conventional organic fluorescent dyes in IHC applications, in practice, quantum dot-conjugated secondary antibodies have proven to be less sensitive than Cy3-, FITC-, and Alexa Fluor-conjugated antibodies, at least in our laboratory. To take advantage of the unique properties of quantum dots while overcoming their relative insensitivity in IHC applications, we developed a TSA detection procedure that uses biotin tyramide deposition followed by incubation with quantum dot-conjugated streptavidin. By carefully adjusting reagent concentrations, we are able to minimize nonspecific signal and achieve sensitive, photostable fluorescence detection (Fig. 5). Multilabel IHC detection can readily be performed by combining biotin tyramide:quantum dot-streptavidin labeling with conventional organic



**Figure 4. Combined TSA and quantum dot IHC detection.** Mouse embryonic spinal cord sections were either left unstained (A) or incubated sequentially with MAP2 primary antibody, HRP conjugated secondary antibody, and biotin-tyramide. The deposited biotin-tyramide was then detected with quantum dot-conjugated streptavidin. Results obtained using quantum dot 565- (B), 585- (C), and 605- (D) conjugates are illustrated. All sections were incubated with Hoechst 33,258 to stain nuclei (blue), and all labeling was visualized using a single filter set consisting of a  $325 \pm 35$ -nm excitation filter, a 370-nm long-pass dichroic beam splitter, and a 400-nm long-pass emission filter. Scale bar equals 100 microns.

## NOTES



**Figure 5. Photostability of quantum dot IHC labeling.** MAP2 immunoreactive neurons in the embryonic mouse spinal cord were detected following biotin-tyramide deposition using either Cy3-conjugated (**A, B**) or quantum dot 605-conjugated (**C, D**) streptavidin. Photomicrographs were taken at time zero (**A, C**) and after 20 min of continuous illumination (**B, D**). The Cy3 signal diminished dramatically over the exposure period (**A** versus **B**), while the quantum dot 605 signal exhibited minimal degradation (**C** versus **D**). Scale bars equal 100 microns.

fluorophore or quantum dot-conjugated antibodies and/or other TSA labeling techniques.

### TSA IHC Detection Method

Our standard TSA IHC detection procedure is outlined below. However, we have previously published detailed protocols for IHC and ISH TSA detection and refer you to those articles and book chapters for more complete information (Zaidi et al., 2000; Roth, 2002; Ness et al., 2003; Akhtar et al., 2007). In addition, several websites with useful information regarding TSA and/or IHC detection include [www.histochem.org](http://www.histochem.org) and [www.neurosciencecore.uab.edu/coreb.htm](http://www.neurosciencecore.uab.edu/coreb.htm).

Whole gestational day 14 embryos and adult mouse brains were obtained from C57BL6/J mice, immersion-fixed overnight at 4°C in Bouin's fixative, and processed for paraffin embedding. Four-micron-thick sections were prepared on a microtome. Following

deparaffinization and rehydration in serial solutions of Citrisolv (Fisher Scientific; Pittsburgh, PA), isopropanol, water, and PBS, endogenous peroxidase activity was inhibited by incubating sections in 3% H<sub>2</sub>O<sub>2</sub> in PBS for 5 min. Sections were then washed in PBS and incubated for 30 min in PBS-blocking buffer (BB; 1% bovine serum albumin, 0.2% powdered skim milk, 0.3% Triton X-100 in PBS) followed by overnight incubation at 4°C with primary antibody diluted in BB. Slides were then rinsed multiple times in PBS and incubated for 1 h at room temperature with HRP-conjugated secondary antibody diluted in BB.

For standard direct TSA detection, fluorophore-conjugated tyramide is diluted in amplification buffer, as recommended by the manufacturer, and applied to the tissue section for 5 to 10 min. For TSA Plus direct detection, the fluorophore-conjugated tyramide can either be diluted in Plus amplification

buffer and incubated on the section for 5 to 10 min as recommended by the manufacturer or, as we prefer, diluted further than suggested but followed by performing the incubation for an extended period of time (30-60 min). For indirect TSA detection, either in the standard or Plus format, hapten-conjugated tyramide (typically biotin-tyramide) is deposited, followed by PBS washes and application of secondary detection reagents (e.g., Cy3-conjugated streptavidin). Sections are then washed in PBS, nuclei counterstained with Hoechst 33,258, and coverslipped in PBS:glycerol (1:1).

### TSA Quantum Dot Detection Method

To maximally utilize the amplification power of TSA and the photostability of quantum dots, we use TSA deposition of biotin-tyramide and quantum dot-conjugated streptavidin. The procedure is identical to that described above for conventional fluorophores, with the exception being that sections are coverslipped in 90% glycerol in PBS. When using quantum dots, it is essential to carefully select appropriate filter sets for viewing IHC signals. Optimal filter sets are recommended by quantum dot manufacturers and can be purchased from a variety of sources. However, depending on the application and quantum dot used in the detection procedure, conventional narrow-band filter sets for commonly used organic fluorophores such as Cy3 or fluorescein can be used. Alternatively, given the fact that quantum dots have a broad excitation range that extends into the ultraviolet, a single filter set consisting of a  $325 \pm 35$ -nm excitation filter, a 370-nm long-pass dichroic beam splitter, and a 400-nm long-pass emission filter can be used to detect individual quantum dot signals or to simultaneously view multiple quantum dot signals.

### TSA tips

- (1) Because TSA depends on the enzymatic activity of peroxidase, endogenous peroxidases may generate antibody-independent IHC signals. To reduce endogenous peroxidase activity, cells or tissue sections can be incubated in  $H_2O_2$  before beginning the IHC detection procedure. The concentration of  $H_2O_2$  and length of incubation will depend on the amount of endogenous peroxidase activity and on tissue fixation. We typically use 0.3%  $H_2O_2$  in PBS for 30 min or 3.0%  $H_2O_2$  in PBS for 5 min. The  $H_2O_2$  may be diluted in  $H_2O$  or methanol, instead of PBS.
- (2) It is essential to minimize nonspecific binding of primary and secondary antibodies to the tissue section. We use a preincubation in PBS-blocking buffer (BB; PBS with 1.0% bovine

serum albumin, 0.2% nonfat powdered skim milk, and 0.3% Triton X-100) and dilute all antibodies in BB.

- (3) The TSA reaction should be optimized for your particular application. Optimization should include testing various concentrations of tyramide conjugate and lengths of the TSA reaction.
- (4) HRP polymer-linked secondary antibodies can provide a dramatic increase in TSA IHC detection sensitivity. We have found that the use of HRP polymers allows a significant reduction in primary antibody concentration and a significant decrease in nonspecific TSA signals.
- (5) The TSA Plus amplification buffer contains a high concentration of salt and may lyse weakly fixed cells. If a precipitating fixative has been used, it may be necessary to refix the tissue in 4% paraformaldehyde before the TSA Plus reaction so as to avoid cell lysis.
- (6) For maximal TSA detection sensitivity, multiple rounds of amplification can be performed. For example, biotin-tyramide deposition can be followed by HRP-conjugated streptavidin and an additional round of fluorophore-conjugated tyramide deposition.
- (7) In all multilabel TSA procedures, it is essential to verify that the HRP enzymatic activity from the first TSA detection procedure has been adequately destroyed before performing a second TSA detection procedure. HRP activity is readily inactivated by 3%  $H_2O_2$  incubation, brief boiling, or most antigen retrieval procedures.
- (8) When performing TSA:quantum dot IHC detection, we have sometimes observed a diffuse nonspecific "haze" when the labeled section is initially viewed with the fluorescence microscope. The haze decreases significantly over a few minutes of continuous illumination. Thus, it is sometimes useful to first expose the section to several minutes of illumination in order to eliminate this background signal before image capture and/or quantitation of fluorescence signal intensity.

## NOTES

**References**

- Adams JC (1992) Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. *J Histochem Cytochem* 40:1457-1463.
- Akhtar RS, Latham CB, Siniscalco D, Fuccio C, Roth KA (2007) Immunohistochemical detection with quantum dots. In: *Methods in molecular biology*, vol 374: Quantum dots: applications in biology (Bruchez MP, Hotz CZ, eds), pp 11-28. Totowa, NJ: Humana Press Inc.
- Bobrow MN, Litt GJ, Shaughnessy KJ, Mayer PC, Conlon J (1992) The use of catalyzed reporter deposition as a means of signal amplification in a variety of formats. *J Immunol Meth* 150:145-149.
- Ness JM, Akhtar RS, Latham CB, Roth KA (2003) Combined tyramide signal amplification and quantum dots for sensitive and photostable immunofluorescence detection. *J Histochem Cytochem* 51:981-987.
- Roth KA (2002) *In situ* detection of apoptotic neurons. Apoptosis. In: *Neuromethods*, Vol. 37: Techniques and protocols (LeBlanc AC, ed.), pp 205-224. Totowa, NJ: Humana Press, Inc.
- Roth KA, Baskin DG (2005) Enzyme-based fluorescence amplification for immunohistochemistry and *in situ* hybridization. In: *Molecular morphology of human tissues* (Hacker GW, Tubbs RR, eds), pp 65-80. Boca Raton, FL: CRC-Press.
- Roth KA, Perry A (2005) Cell and tissue imaging techniques. In: *Translational and experimental medicine* (Schuster D, Powers W, eds), pp 396-412. Philadelphia: Lippincott, Williams & Wilkins.
- Shindler KS, Roth KA (1996) Double immunofluorescent staining using two unconjugated primary antisera raised in the same species. *J Histochem Cytochem* 44:1331-1335.
- Watson A, Wu X, Bruchez M (2003) Lighting up cells with quantum dots. *BioTechniques* 34:296-303.
- Wu X, Liu H, Liu J, Haley KN, Treadway JA, Larson JP, Ge N, Peale F, Bruchez MP (2003) Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots. *Nature Biotech* 21:41-46.
- Zaidi AU, Enomoto H, Milbrandt J, Roth KA (2000) Dual fluorescent *in situ* hybridization and immunohistochemical detection with tyramide signal amplification. *J Histochem Cytochem* 48:1369-1376.