

# Preembedding Immunolectron Microscopy: Applications for Studies of the Nervous System

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**Abstract:** This chapter addresses the basic applications of tract-tracing and preembedding immunoperoxidase and immunogold–silver labeling for transmission electron microscopy, focusing primarily on identifying the cellular and subcellular localization of proteins of relevance to neurotransmission and on defining synaptic connectivity within neuronal circuits. Information is provided regarding the use of preembedding immunoperoxidase and immunogold techniques to identify the cellular and subcellular localization of neuronal receptors and transporters. The chapter also describes in detail a triple-labeling approach designed by our laboratory for identifying synaptic inputs to neuronal cell populations defined both by their projection targets and by their transmitter phenotype. Protocols presented in the Appendix are designed to enable researchers trained in small animal surgery, immunocytochemistry, electron microscopy, and appropriate laboratory safety procedures to perform ultrastructural investigations similar to those described here.

**Keywords:** electron microscopy, immunocytochemistry, immunogold, immunoperoxidase, preembedding, tract-tracing, ultrastructure

## I. INTRODUCTION

Having been available for 70 years, it seems reasonable to ask what transmission electron microscopy (TEM) can add to the investigation of the nervous system in the new millennium. Today, the basic synaptic organization of most brain regions is known, as is the morphological detail of most neurons and support cells. Chapters in previous volumes of this series have addressed the applications of TEM for studies of brain/neuronal structure, patterns of degeneration, and identification of synaptic connectivity using tract-tracing, immunocytochemistry, and electrophysiological cell filling (Heimer and Robards, 1981; Heimer and Zaborszky, 1989). In the chapter by Wouterlood of the current volume, a sophisticated technique for using confocal microscopy to define synaptic contacts is described, raising further questions about how long TEM will endure as a staple approach for neuroscience investigation. Is it the case that TEM will increasingly become a legacy method used only by aesthete scholars eager to have quality photomicrographic evidence at the highest magnifications possible?

Obviously, we believe that this is not the case and that TEM, in combination with procedures to identify discrete pathways and proteins at cellular and subcellular levels, is as powerful and up to date as ever. Of course, TEM is not without its disadvantages; it is expensive, time-consuming, requires considerable technical skill, and is capable of generating false-positive and false-negative results, the latter even in the hands of experienced researchers. In the current chapter, we will present a general assessment of the progress made in TEM studies of the nervous system since the last volume of this series and provide technical and interpretational guidelines that are critical for planning modern TEM experiments. We will briefly characterize relatively common procedures and refer readers to other chapters where these are described in greater detail. In addition, we will supply sometimes

hard-to-find information that has not yet been described in this series or needs to be updated. This chapter focuses on preembedding immunocytochemical methods for TEM. The reader is referred to the chapter by Mathisen *et al.* (this volume) for a description of postembedding techniques that are also used for immunoelectron microscopy.

## II. APPLICATIONS

### A. General Applications and Appraisal of the Methods

The higher magnification and resolution afforded by the TEM method of analysis presents distinct advantages compared to light microscopic (LM) techniques. TEM allows the observation of cell ultrastructure and how this morphology changes with natural or extrinsically imposed events such as aging, drug treatment, or stress. For such questions, the application of LM and Golgi impregnation is also quite useful, particularly for measuring dendritic branching and for counting spine density on identified cell types (Irwin *et al.*, 2002; Kolb *et al.*, 2003). However, TEM in combination with unbiased stereological measurements can more accurately and more quickly provide counts of overall spine number in a given brain region, as well as the number of axon terminals, dendrites, and synapses in that area. As such, TEM application of the physical disector method has become an important tool for analyzing how structure and synaptology change with experience. This methodology can also be combined with immunocytochemistry (Beaulieu *et al.*, 1994). Such applications will not be addressed here, and the reader is directed to the existing literature on physical disector methods for counting neuronal elements (Howard, 1990; Mayhew, 1992; von Bartheld, 2002) and to the chapter by Avendaño in this volume.

With relatively few exceptions, most antigens can be immunolabeled by both LM and TEM, although the latter allows for a more precise assessment of the subcellular position of antigens, and the relationship of labeled structures to each other can be determined more precisely with TEM techniques. This makes the latter method essential for the most definitive determination of the cellular and subcellular distribution of neurotransmitters, receptors, transporters, enzymes, metabolites, and so on (see section “Ultrastructural Immunolocalization of Neurobiological Proteins”). TEM is also suitable for more recent studies of protein trafficking (Bloch *et al.*, 2003; Ravary *et al.*, 2001), although LM approaches have the advantage of being able to follow these intracellular processes in real or near-real time.

TEM allows the visualization of the fine characteristics of synapses occurring between neuronal structures identified by tract-tracing or immunocytochemistry. Hence, it continues to find utility as a tool for studying communication between functional neural systems. For this particular application, TEM approaches have gained in analytical power since the last release of neuroanatomical tract-tracing methods, due to the ability to recognize at least three markers on the same sections. Although LM techniques such as

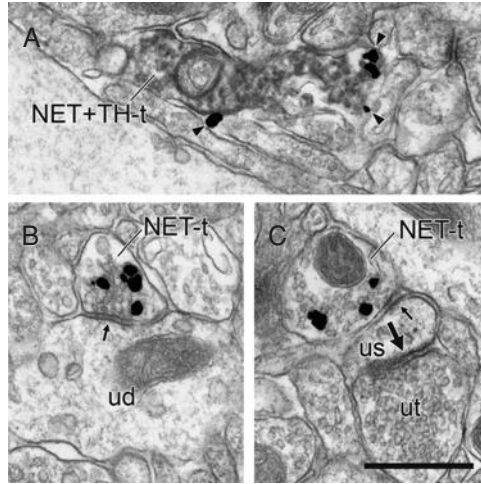
that described in the chapter by Wouterlood (this volume) may eventually provide comparable information, TEM will continue to represent the principal method for detailed synaptic connectivity studies of neuronal circuitry. Below, we address in more detail the important technical issues that deal with the studies of this type.

## **B. Ultrastructural Immunolocalization of Neurobiological Proteins**

The functional impact of any given neurotransmitter is determined by the activation of receptor subtypes with particular affinities and activities and often by the removal of the transmitter from the extracellular space by plasma membrane transporters. Although these general principles of neurotransmission apply to most neuronal systems, differences in the extent to which receptors and transporters are inserted in the plasma membrane can cause distinct cellular responses to neurochemicals that are highly regulated. Hence, a powerful application of ultrastructural immunocytochemistry is the localization of neurobiologically relevant proteins (Cornea-Hebert *et al.*, 1999; Ferguson *et al.*, 2003; Garzón *et al.*, 1999; Glass *et al.*, 2004; Hanson and Smith, 1999; Huang and Pickel, 2002; Pickel and Chan, 1999; Pickel *et al.*, 2004; Miner *et al.*, 2000, 2003a, 2003c; Riad *et al.*, 2000). The information obtained from ultrastructural descriptions of their distribution is crucial to our understanding of the operation of these systems.

### **1. General Issues of Pre- and Postembedding Immunocytochemical Methods**

The main techniques used for immunologically based electron-dense staining of these proteins are preembedding immunoperoxidase, preembedding immunogold–silver, and postembedding immunogold. These are “indirect” methods of labeling that involve a “primary” unconjugated antibody directed against the antigen of interest. The primary antibody is an unmodified protein that cannot be visualized by TEM. Therefore, the presence of the primary antibody is detected by using a secondary antibody that is directed against species-specific antigenic sites on the primary antibody. This secondary antibody is conjugated to a molecule that will allow the creation of an electron-dense product that can be readily visualized. Some of the labeling molecules are enzymes whose reaction product can be detected by the addition of a substrate, whereas others are electron-dense heavy metals that can be visualized directly. Because the end reaction products from the various immunocytochemical techniques possess distinguishing characteristics, a combination of the labeling methods can be used to identify multiple proteins and determine their cellular or subcellular relationships to each other. For instance, preembedding immunoperoxidase labeling of one antigen can be followed by preembedding immunogold–silver (Fig. 2.1A) or postembedding immunogold labeling of another antigen. Also, both



**Figure 2.1.** Electron micrographs showing axon terminals in the rat PFC singly labeled by immunogold–silver for NET (NET-t) or dually labeled for NET and by immunoperoxidase for TH (NET + TH-t). Axons dually labeled for NET and TH contain the majority of gold–silver particles along the plasma membrane (arrowheads in A). Axons singly labeled for NET contain immunogold–silver particles mainly within the cytoplasm and occasionally form symmetric synapses (small arrows) onto unlabeled dendrites (ud in B) or spines (us in C). In (C), the spine also receives an asymmetric synapse (large arrow) from an unlabeled terminal (ut). Scale bar represents 0.5  $\mu\text{m}$ .

preembedding (Yi *et al.*, 2001) and postembedding (see chapter by Mathisen *et al.*, this volume) immunogold procedures can make use of different-sized gold or gold–silver particles to perform dual-labeling studies.

For the preembedding techniques, the immunocytochemical labeling is performed on thick sections (50  $\mu\text{m}$ ), prior to embedding in the plastic resin necessary for cutting the ultrathin (60 nm) sections used for TEM. These preembedding techniques allow satisfactory visualization of most proteins and are becoming somewhat standardized, as described in detail in the Appendix. However, proteins situated in areas that may be inaccessible to antibodies are not consistently recognized with these methods. Such locations include dense membrane barriers like the synaptic complex (e.g., many receptors and transporters) or within vesicles (e.g., the synthetic enzyme dopamine  $\beta$ -hydroxylase).

For the postembedding procedure, immunocytochemistry is performed after the thick sections are embedded in plastic and sliced into ultrathin sections. Due to the thinness of the sections, the experimenter can be confident that antibodies contact most portions of any given cell. Therefore, the major advantage of the postembedding technique is that it allows the visualization of proteins within areas that are typically not accessible with the preembedding procedures. For instance, receptors located within pre- and postsynaptic densities can be visualized (Hanson and Smith, 1999; Nusser *et al.*, 1995). However, many antigens become denatured during

the embedding process, rendering this technique unfeasible for the localization of many proteins. Another potential disadvantage is that postembedding gold reagents may interact nonspecifically with the surface of ultrathin sections, resulting in background staining and interfering with unequivocal identification of specifically labeled structures (Van Haeften and Wouterlood, 2000). For a complete review of the applications and technical considerations regarding postembedding methods, the reader is referred to the chapter by Mathisen *et al.* (in this volume).

## 2. Preembedding Immunoperoxidase Labeling

The preembedding immunoperoxidase technique involves localizing horseradish peroxidase enzyme activity. The horseradish peroxidase molecule is easily conjugated to antibodies, and because it is relatively small, it can readily penetrate membranes of cells fixed with aldehydes. When the peroxidase molecule is exposed to a hydrogen peroxide substrate and the capturing agent 3,3'-diaminobenzidine (DAB), the oxidation products of the DAB will form a brown reaction product. This insoluble product becomes electron dense following chelation by osmium tetroxide (Fig. 2.1A).

### a. Strengths and Weaknesses

The major benefit of the immunoperoxidase technique is its superior level of sensitivity. This is particularly true given the various techniques for signal amplification with this method (Hsu *et al.*, 1981; Ordroneau *et al.*, 1981). Compared to the preembedding immunogold approach (described below), immunoperoxidase maximizes the labeling of antigen (Chan *et al.*, 1990), which presents a considerable advantage if the amount of the protein of interest in a given structure is low. Hence, if the desired end point is to demonstrate that a particular neuronal element contains a particular protein, this is the method of choice.

The main disadvantage of immunoperoxidase labeling is that the catalytic activity of the enzyme can result in the accumulation of reaction product that is capable of diffusion away from the original site of activity and, thus, the location of the antigen (Novikoff *et al.*, 1972). In this case, immunoperoxidase is not the best choice if the goal of the study is to determine the exact subcellular location of a protein. Another potential disadvantage of immunoperoxidase is that the accumulation of excessive amounts of reaction product can obscure the visualization of cytoplasmic organelles and synaptic specializations, which limits the determination of cellular structure or the presence of synaptic contacts, and challenges the identification of synapse type. In some cases, the crystalline reaction product can become sufficiently excessive to break through membranes and leak into surrounding structures. Consequently, one must take great care when considering structures lying immediately adjacent to heavily labeled profiles to ensure that all membranes that separate the profiles are intact.

Another potential drawback of immunoperoxidase staining is that some cellular elements contain proteins with endogenous peroxidase-like activity. Such activity is present in red blood cells. As a result, suboptimal perfusions that leave behind many blood cells within brain capillaries can be problematic, in that they have the capacity to use up reagents intended for immunoperoxidase labeling with subsequent reduction in the density of specific labeling. In addition, some glial cells and even some neurons appear to have endogenous peroxidase-like activity (Conradi, 1981; Srebro, 1972; Svensson *et al.*, 1984). Therefore, one may wish to incubate tissue sections in hydrogen peroxide (see Appendix, section “Sodium Borohydride and Hydrogen Peroxide Treatments”) prior to immunocytochemical procedures in order to quench this endogenous peroxidase activity.

### **b. Applications and Analysis**

The immunoperoxidase technique is well suited for qualitative analysis of the distribution of proteins. In other words, the *presence* or *absence* of a protein can be readily determined using this labeling procedure (Fig. 2.1A). Moreover, if the aim of an experiment is to establish the general localization of a protein (e.g., expression in soma, dendrites, or axons), this procedure is optimal, given its sensitivity. Quantitative analysis of immunoperoxidase-labeled tissue is typically limited to counting the number of labeled structures within a given area or volume. Because the peroxidase reaction product is capable of spreading over some distance, determination of the subcellular localization of proteins is imprecise with this technique. Therefore, if knowledge of exact protein location is desired, this procedure is not the most viable choice. Likewise, the density of antigens within labeled structures cannot be discerned with precision, although estimates of light or heavy localization are possible. It should be noted that this method is valuable as a first step in localizing proteins. Because immunoperoxidase labeling is the most sensitive immunocytochemical technique and the least costly in terms of time and money, it is practical to use this technique to gain knowledge about the overall amount of an antigen and its general distribution in order to guide further labeling with less sensitive techniques. Some proteins, particularly those in low abundance, often do show “hot spots” of immunoreactivity that strongly suggest a certain subcellular pattern of localization (Aoki *et al.*, 2001; Doly *et al.*, 2004; Mi *et al.*, 2000). Hence, this may be the best first approximation that is available in the event that the less sensitive immunogold–silver methods do not produce consistently detectable labeling.

### **3. Preembedding Immunogold–Silver Labeling**

Immunogold labeling exploits the conjugation of colloidal gold particles to immunoglobulins (DeMey and Moeremans, 1986). Therefore, the tissue is exposed to the primary antibody and then to a secondary antibody that

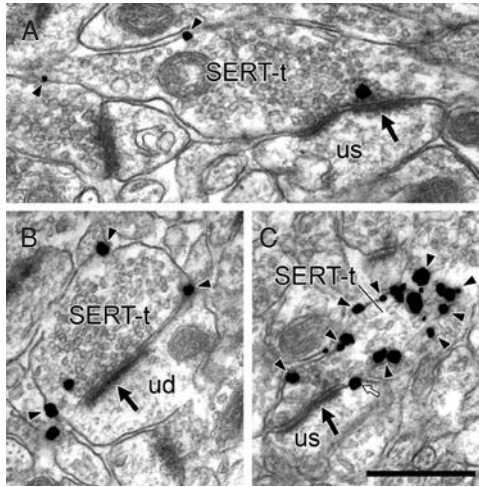
has been conjugated to a gold particle. Colloidal gold probes may be prepared in a variety of sizes (from  $\sim 1$  to 150 nm). For electron microscopy, gold probes below 30 nm are typically used. For example, it has been shown that gold particles in the range of 10 nm can be used without silver enhancement for preembedding subcellular immunolocalization (Zaborszky *et al.*, 2004). However, the use of ultrasmall gold particles ( $\sim 1$  nm) conjugated to secondary antibodies has the advantage of greater tissue penetration for preembedding studies (Chan *et al.*, 1990; Pickel *et al.*, 1993). Because particles of this size are not readily visualized by electron microscopy, the gold is then enlarged by allowing silver to complex with it. It should be noted that, because antibody penetration is not an issue for postembedding immunolabeling, the gold particles conjugated to the secondary antibodies used for that method are much larger (10–20 nm), and can be easily distinguished by electron microscopy without further enhancement procedures.

### a. Strengths and Weaknesses

In contrast to the precipitates formed in immunoperoxidase staining, silver-enhanced gold particles are more electron dense, more discrete, and spherical (Figs. 2.1–2.3). Hence, they are quite easily identified. Their discrete nature and the fact that the gold particle remains attached to the secondary antibody means that diffusion away from the source of the antigen is not a problem as it can be for immunoperoxidase. Thus, the predominant strength of this technique is that it permits a more precise view of antigen localization (Figs. 2.1–2.3). For instance, this method will distinguish whether a protein is inserted within the plasma membrane or within the cytoplasm (see especially Fig. 2.1) (Ferguson *et al.*, 2003; Garzón *et al.*, 1999; Miner *et al.*, 2000, 2003c; Pickel and Chan, 1999). It is possible that the silver-enhanced gold particles will settle at areas that are slightly removed from the antigen. However, the distance in question is typically in the range of 20–25 nm (Paspalas and Goldman-Rakic, 2004) (e.g., along the inner or the outer plasma membrane). If a more refined level of protein localization is required, postembedding immunogold labeling may be necessary (see the chapter by Mathisen *et al.*). Having discrete particles also allows for a certain degree of quantification with the immunogold–silver method. Particles can be counted, measured as number per unit area or per unit volume, and assigned to different compartments such as cytoplasmic or plasmalemmal.

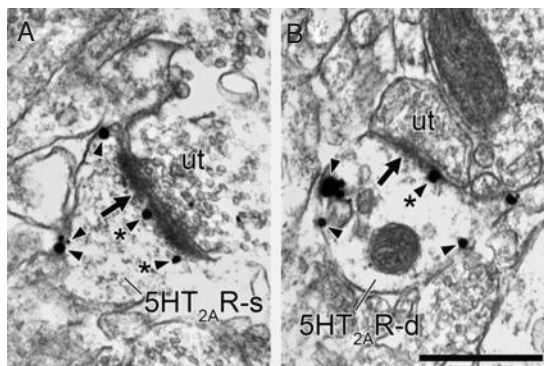
Although the preembedding immunogold–silver technique is a powerful method for localizing proteins, it has limitations. One weakness is that the level of labeling achieved for a given amount of antigen is generally lower than with immunoperoxidase. It has been estimated that the immunogold–silver approach is perhaps one order of magnitude less sensitive than immunoperoxidase (Chan *et al.*, 1990). This is likely due to decreased penetration into the tissue section of the secondary antibody with the gold particle conjugated to it and the absence of methods for signal amplification.





**Figure 2.2.** Electron micrographs of the rat PFC showing immunogold–silver labeling for SERT in axon terminals (SERT-t) forming asymmetric synapses (large arrows) onto unlabeled dendrites (ud) or spines (us). The majority of gold–silver particles for SERT are located along the plasma membrane (arrowheads), although some are also distributed within the cytoplasm. Occasionally, gold–silver particles for SERT are found in close proximity to sites of synaptic contact (white arrow). Scale bar represents 0.5  $\mu\text{m}$ .

Another possible weakness is that preembedding immunogold may be better suited to detecting plasmalemmal antigens when they are located at extrasynaptic than at synaptic sites (Figs. 2.2 and 2.3) (Bernard *et al.*, 1997). Some studies indicate that preembedding immunogold is unable to detect



**Figure 2.3.** Electron micrographs from the rat PFC illustrating immunogold–silver labeling for  $5\text{HT}_{2A}$  receptors ( $5\text{HT}_{2A}$  R) in dendritic shafts ( $5\text{HT}_{2A}$  R-d) and spines ( $5\text{HT}_{2A}$  R-s) receiving synaptic input (arrows) from unlabeled terminals (ut). Arrowheads indicate immunogold–silver particles associated with the plasma membrane. Those with asterisks are within or near the postsynaptic density. Scale bar represents 0.5  $\mu\text{m}$ .

proteins that are embedded with in the synaptic complex, including the synaptic cleft (Baude *et al.*, 1995; Bernard *et al.*, 1997). However, other published studies show acceptable labeling of synaptic proteins (Chen *et al.*, 2004; Kulik *et al.*, 2003; Wong *et al.*, 2002) (see also Fig. 2.3). Conversely, the postembedding immunogold method is considered the preferred method for visualizing proteins within synaptic specializations (Bernard *et al.*, 1997). Most likely, this is due to the fact that the synaptic complex has been sectioned at 60 nm, exposing antigenic sites within the complex. Another potential weakness of the preembedding immunogold–silver technique is that the silver enhancement of the gold particles is sensitive to several experimental factors, and therefore care must be taken to obtain reliable gold–silver labeling (see section “Choice of Markers”).

## b. Applications and Analysis

The ultrastructural immunolocalization of neurobiological proteins is a powerful method for identifying the subcellular distribution of receptors and transporters that may or may not be situated at predicted locations (Ferguson *et al.*, 2003; Hanson and Smith, 1999; Masson *et al.*, 1999). Regardless of whether such studies match or deviate from expectations, they elucidate the anatomical substrates for the actions of neurotransmitters.

For example, we have utilized the preembedding immunogold–silver method to investigate the subcellular localization of monoamine transporters in the rat cortex (Miner *et al.*, 2000, 2003c). In the case of the serotonin transporter (SERT), immunogold–silver particles are typically found on the plasma membrane in extrasynaptic locations (Fig. 2.2). Occasionally, particles are localized close to the synaptic specialization (Fig. 2.2C). Surprisingly, the norepinephrine transporter (NET) is localized predominantly in the cytoplasm (~75%), with only a minority of gold–silver particles found along the plasma membrane (Fig. 2.1). We have also performed a preliminary investigation (unpublished data) of the subcellular localization of the serotonin (5-hydroxytryptamine, 5HT) 2A receptor subtype (5HT<sub>2A</sub>R) in cortical cells utilizing an antibody that was used previously to localize this receptor by immunoperoxidase (Miner *et al.*, 2003a). The preembedding gold–silver method shows that this receptor is localized to both extrasynaptic and synaptic plasmalemmal sites (Fig. 2.3).

In addition to characterizing the typical distribution of neurobiologically relevant proteins, these techniques allow the provocative documentation of changes following physiological or pathological treatments. For instance, alterations in the subcellular distribution of various proteins have been observed in response to genetic, pharmacological, or environmental manipulations (Dumartin *et al.*, 1998, 2000; Glass *et al.*, 2004; Miner *et al.*, 2003b, 2004; Nusser *et al.*, 1999; Riad *et al.*, 2004). The findings from such studies provide valuable insights into the function and trafficking of these important proteins.

For analysis of tissue in which the subcellular localization of gold–silver particles, and hence antigen, is the dependent measure, it is customary to count the total number of gold particles in the structure of interest, and then to assign each gold particle to a particular compartment (e.g., cytoplasm, smooth endoplasmic reticulum, vesicle, plasma membrane). One can then assess as a percentage the tendency of gold particles to accumulate within a particular compartment. It is also possible to determine whether gold particles are localized to the inside or outside edge of a membrane, although as mentioned earlier, the exact position of the relatively large preembedding immunogold–silver particles may not reflect the antigen distribution with perfect fidelity. More refined localization requires postembedding immunogold methods.

#### 4. Dual-Labeling Procedures

A major strength of the immunocytochemical procedures discussed here is that several procedures may be combined in order to visualize two antigens in the same tissue section. The type of dual-labeling method used most frequently in our laboratory is preembedding immunoperoxidase and immunogold–silver. The immunoperoxidase reaction product is dense, dark, and flocculent, whereas the silver-enhanced gold particles are fully black and particulate. Therefore, the two types of markers can be readily distinguished, even in the same structure. In our experience, we prefer to use a “parallel” labeling method in which the tissue is incubated simultaneously in the primary antibodies for both antigens. Then, the immunoperoxidase labeling is performed, followed by the immunogold–silver procedures. The order of manipulations can also be switched so that immunogold–silver is performed first (Katona *et al.*, 2001). However, in this case, an additional step of gold toning may be needed to stabilize the immunogold–silver prior to peroxidase treatments (Arai *et al.*, 1992). Either dual-labeling combination necessitates that the primary antibodies be raised in different species, but it allows the experimenter to determine the presence of one antigen and the precise subcellular localization of the other. For example, in our studies of the localization of NET in the cortex, we found that axons containing immunoperoxidase labeling for the catecholamine synthetic enzyme, tyrosine hydroxylase (TH), contain preembedding immunogold–silver labeling for NET primarily along the plasma membrane (Fig. 2.1A) (Miner *et al.*, 2003c). This is in distinct contrast to axons that contain NET and not TH (Fig. 2.1B,C), where NET is predominantly cytoplasmic.

Other TEM methods for labeling two antigens in the same tissue sections include combinations of immunoperoxidase chromogens with different physical characteristics (Charara *et al.*, 1996; Norgren and Lehman, 1989; Smith *et al.*, 1994; Zaborszky and Heimer, 1989; Zhou and Grofova, 1995) (see section “Choice of Markers”) and combinations of preembedding immunogold–silver techniques (Yi *et al.*, 2001). For the latter procedure,

silver intensification of one antigen is performed, followed by silver intensification of a second antigen. The labeling for each can be distinguished based on the size of the gold–silver particles, with the particles for the first antigen being larger, because they are exposed to two enhancement sessions. This procedure allows the subcellular localization of two antigens within the same tissue.

Finally, two antigens can be visualized simultaneously by combining pre-embedding immunoperoxidase or immunogold–silver with postembedding immunogold procedures. An advantage of this technique is that two primary antibodies that were generated within the same species can be used, because the osmication and dehydration performed prior to plastic embedding destroys the antigenicity of the first primary antibody. However, it should be noted that this is feasible only if those same steps do not denature the antigen to be labeled by the postembedding method (see above).

### C. Analyses of Synaptic Connections

In order to determine whether neurons of interest are synaptically connected, it is necessary to distinguish particular populations of cells based on their specific protein content and/or on their topographic projections. Although neurochemical phenotypes can typically be defined in naive animals, the identification of neuronal connections requires surgical intervention to introduce tract-tracing agents, at least at present (see section “Prospects for the Future”). Here, we discuss the principles of study design that combine tract-tracing and neurochemical definitions with TEM analysis.

It is beyond the scope of this chapter to compare the effectiveness and methodology for all the available anterograde and retrograde tracing approaches, many of which have been described in previous volumes of this series (Gerfen *et al.*, 1989; Pickel and Milner, 1989; Skirboll *et al.*, 1989; Steward, 1981; Warr *et al.*, 1981; Zaborszky and Heimer, 1989). Relevant information regarding different methods of intraparenchymal injections, including iontophoretic and pressure injections have also been covered earlier (Alheid *et al.*, 1981). Here, we will focus on the tracers that are used most commonly in ultrastructural studies, and particularly those that combine well with each other and with techniques to label phenotypic markers. The main requirements for such tracers are (1) sensitivity in labeling as many of the neurons and processes that contribute to a pathway as possible, (2) specificity in the direction of transport, (3) recognizable filling of the parts of neurons that will be examined by TEM, (4) compatibility with ultrastructural preservation, and (5) compatibility with other markers. If multiple tracers need to be combined, the choices may become more limited. In order to avoid the performance of multiple survival surgeries, we use tracers with similar optimal survival times. The clear understanding of individual tracer competence is a critical issue for planning TEM experiments, and the inability of any particular tracer or combination of tracers to

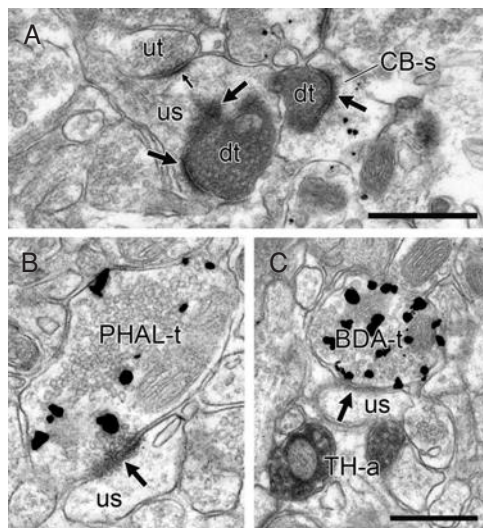
fulfill all of the above requirements is the main reason why the results may need to be verified by alternative tracer approaches.

## 1. Anterograde Tract-Tracing

### a. Anterograde Degeneration

The induction of anterograde degeneration following brain lesion is the oldest tracing method available, but it still has excellent utility for some investigations. The three main lesion approaches are physical disruption (e.g., suction), cytotoxic injection, and electrolytic lesion (Moore, 1981), with cytotoxic injections having the advantage of being directed only to cell bodies and dendrites in the region while sparing fibers of passage. Physical disruption and electrolytic lesions provide a more certain disruption of all cellular elements at the same time, and the electrolytic approach can be calibrated to control the degree of current spread. After a survival time that must be determined empirically for each pathway, the axon terminals of the lesioned neurons will show signs of degeneration that can be readily distinguished in the electron microscope (Mugnaini and Friedrich, 1981). In the best case, the terminals will demonstrate the characteristics of electron-dense degeneration (Fig. 2.4A): shrunken and distorted boundaries, darkened cytoplasm, disrupted organelles like mitochondria and synaptic vesicles, and frequent engulfment on nonsynaptic sides by glial processes (Mugnaini and Friedrich, 1981; Sesack and Pickel, 1992). Despite this extensive disruption of structure, axon terminals will retain their synaptic connections with target cells until they are completely removed by glia, and this provides the degeneration method its utility in studies of synaptic connections.

Anterograde degeneration has two major advantages and several disadvantages of note. First, in our experience, it is the most effective method of anterograde tracing for TEM in the rodent, assuming that disruption of fibers of passage is not an issue for the pathway under study (Sesack and Pickel, 1992). Depending on the size of the lesion, many of the axons of interest will be affected. Moreover, the method does not require any chemical or immunolabeling, and affected terminals will be observed throughout the thickness of ultrathin sections. This is in contrast to tracers that require immunocytochemical detection, whose recognition will be limited to the tissue plastic interface (see section “General Sampling Issues”). Second, anterograde degeneration can be combined with immunocytochemical methods, including sensitive immunoperoxidase approaches (e.g., avidin-biotin peroxidase; ABC (Hsu *et al.*, 1981) to label target neurons or convergent structures so that the two methods together provide excellent likelihood of finding hypothesized synaptic connections (i.e., low false-negative rate). In this regard, it should be noted that the two methods can be further combined with preembedding immunogold-silver for triple-labeling studies.



**Figure 2.4.** Electron micrographic examples of anterograde tract-tracing in the rat forebrain. (A) Two degenerating axon terminals (dt) in the PFC following an electrolytic lesion in the mediodorsal thalamus form asymmetric synapses (large arrows) onto spines either labeled by immunogold–silver for calbindin (CB-s) or unlabeled (us). The latter receives an additional symmetric synapse (small arrow) from an unlabeled terminal (ut). (B, C) Axon terminals in the amygdala (B) or NAc (C) containing immunogold–silver labeling for PHA-L (PHA-L-t in B) or BDA (BDA-t in C) transported anterogradely from the PFC or paraventricular thalamus, respectively, synapse onto unlabeled spines. In (C) immunoperoxidase labeling for TH is evident within axons (TH-a) in the adjacent neuropil. Scale bar in (A) represents 0.5  $\mu\text{m}$  for (A) and (B); scale bar in (C) represents 0.5  $\mu\text{m}$  for (C).

There are several disadvantages to the anterograde degeneration method:

1. The correct survival time after lesion is critically important. At short times, it will be hard to recognize terminals undergoing degeneration, whereas at long times, synaptic contacts will be disrupted and the affected terminals will be eliminated. Deviations of a half day can make a difference, so that the optimal timing for a particular pathway must be established empirically.
2. The method is not applicable for all connections or in all species. Monoamine pathways are particularly difficult to label with this approach, and early studies that attempted to use the method produced erroneous results. A detailed analysis of the difficulties associated with anterograde degeneration for detection of monoamine axons is available (Zaborszky *et al.*, 1979). In addition, not all pathways appear to undergo a process of anterograde degeneration that can be easily detected by TEM. For example, in a prior study of efferents from the prefrontal cortex (PFC), we were able to label the projection to the nucleus accumbens (NAc) using anterograde degeneration but not

the fibers known to innervate the ventral tegmental area (VTA), despite examination of lengthy survival times (Sesack and Pickel, 1992). With regard to other species, the rat is particularly amenable to the anterograde degeneration method because of its small size and the ease of lesioning the majority of a pathway nearly simultaneously. In primates, the anterograde degeneration process can proceed at different rates in different neurons, making the selection of an optimal survival time nearly impossible.

3. False-positive results can be generated if the lesion disrupts fibers not originating from but passing through the lesioned area en route to the target of interest.
4. Anterograde degeneration cannot be used for phenotypic characterization of afferents. Such features as size, normal morphology, and immunoreactivity for particular markers will be compromised.

#### **b. *Phaseolus vulgaris* Leucoagglutinin (PHA-L)**

PHA-L is a plant lectin that has many superior properties for anterograde tract-tracing, including specificity for the anterograde direction and the ability to fill the processes of neurons that take it up in a Golgi-like fashion (Gerfen and Sawchenko, 1984; Wouterlood and Groenewegen, 1985; Wouterlood and Jorritsma-Byham, 1993). A detailed description of the application of PHA-L tract-tracing can be found in Gerfen *et al.* (1989), and combination of this method with transmitter identification in postsynaptic targets has been described in a previous chapter in this series (Zaborszky and Heimer, 1989). Here, we will focus primarily on the advantages and disadvantages of this tracer for TEM studies.

The requirement of PHA-L for iontophoretic injection to stimulate uptake is both an advantage and a disadvantage of this method. Iontophoresis is advantageous because it contributes to the selective uptake into soma and dendrites and, hence, the anterograde specificity. It also helps to create the smaller injection sites that are characteristic of this tracer, which may be desirable or undesirable, depending on the target of interest. Moreover, the equipment for iontophoretic application is somewhat expensive, and so this can limit the availability of the PHA-L method.

Regarding other advantages, PHA-L rarely undergoes retrograde transport and only in certain systems (Shu and Peterson, 1988), making it an otherwise exclusive anterograde tracer for most pathways. Another important advantage is that it is not taken up by fibers of passage. To date, there are no reports of PHA-L failing to label any particular pathway. Furthermore, PHA-L remains within the neurons that take it up for considerable periods of time (up to 10 weeks) (Wouterlood *et al.*, 1990), facilitating studies in which the introduction of the tracer and sacrifice of the animal must be separated by some period of time. In the early stages after injection of PHA-L, the extent of anterograde transport increases with time, so that for TEM studies, it is generally advisable to wait 10–14 days following injection.

PHA-L is transported into all branches of an axon, making it possible to use this tracer to study clearly identified local collaterals within the region of injection. Although biotinylated dextran amine (BDA) has the same ability (Melchitzky *et al.*, 2001), its greater potential to undergo retrograde transport and trafficking into the collateral branches of retrogradely labeled cells (Wouterlood and Jorritsma-Byham, 1993) (see below) may compromise its usefulness for studying local collaterals. Finally, the PHA-L method can be combined with: BDA as a second anterograde tracer (Dolleman-Van der Weel *et al.*, 1994; French and Totterdell, 2002, 2003), retrograde tract-tracing agents, or immunocytochemistry to identify target phenotype. In this regard, PHA-L can be localized by either immunoperoxidase or immunogold–silver methods (Fig. 2.4B), with the other marker being used to label additional tracers or transmitters.

The PHA-L method has two principal disadvantages:

1. It is reported to have less sensitivity compared to BDA (Wouterlood and Jorritsma-Byham, 1993), although we have not found this difference to be particularly remarkable in our own studies of brain connectivity. Lower sensitivity is, in part, due to the relatively small injection sites achieved with the iontophoretic technique. However, the major reason for lower sensitivity is the requirement for immunolabeling to visualize PHA-L. This lowers the sensitivity for TEM studies in which ultrastructural preservation and penetration of the tissue surface must be carefully balanced (see section “General Sampling Issues”).
2. Small injection sites limit the usefulness of the method for studies in primates. Of course, PHA-L is the preferred method when injections must be confined to a small target.

### c. Biotinylated Dextran Amine (BDA)

BDA (10,000 MW) is another example of a highly successful method for anterograde tract-tracing (Brandt and Apkarian, 1992; Reiner *et al.*, 2000; Veenman *et al.*, 1992), as described in more detail in the chapter by Reiner and Honig. The tracer can be injected as a 10% solution in 0.01 M phosphate buffer, pH 7.4, either by iontophoresis or by pressure, enabling the investigator to make large or small injections as desired. The ability to make large injections is particularly important for tract-tracing in primates. BDA utilizes a simple detection method, in which brain sections are simply incubated in ABC reagents (Hsu *et al.*, 1981) without the need for immunolabeling procedures. This provides BDA with greater sensitivity than PHA-L (Wouterlood and Jorritsma-Byham, 1993). However, when needed, BDA can be labeled by immunogold–silver methods (Fig. 2.4C) using an antibody directed against biotin (Pinto *et al.*, 2003).

At the same time, BDA presents with some rather significant disadvantages. Specifically, it can be transported retrogradely and via fibers of passage (Reiner *et al.*, 2000; Van Haeften and Wouterlood, 2000). Moreover, the



BDA that undergoes retrograde transport can subsequently travel into the collaterals of these neurons and give rise to false-positive results when used for anterograde tracing (Chen and Aston-Jones, 1998; Reiner *et al.*, 2000). This requires that investigators check carefully for retrograde transport of BDA into any brain area that is also afferent to the region of interest.

## 2. Retrograde Tract-Tracing

For LM studies, it is usually sufficient that retrograde tracers transport into the soma and proximal dendrites of labeled cells. However, for TEM studies of synaptic inputs to identified cell populations, there may be the added requirement that a retrograde tract-tracing agent also traffic into the distal dendrites of neurons, if the afferent under study synapses distally. This poses one of the most difficult challenges in studies of synaptic connectivity. To our knowledge, only a few tracers possess this ability, and then only in certain cells or under special circumstances. Here, we review three tracers that have been reported to infiltrate distal dendrites following retrograde transport. A fourth retrograde tracer, cholera toxin subunit B, has also been reported in the literature to show usefulness in ultrastructural analyses, and interested readers are directed to several reviews on this subject (Bruce and Grofova, 1992; Ericson and Blomqvist, 1988; Llewellyn-Smith *et al.*, 1990). In our hands, we have observed no obvious advantages with cholera toxin over the tracers that follow.

### a. BDA

A smaller molecular form of BDA (3000 MW) is available for use as a retrograde tracer that can be delivered either by pressure or by iontophoresis using a different buffer: 10% solution in 0.1 M sodium citrate-HCl (Reiner *et al.*, 2000). Like the use of BDA for anterograde tract-tracing, large injections of BDA can be made, making this technique useful for primate studies. Retrogradely transported BDA can be detected with a simple ABC method, and there is no evidence that it undergoes transneuronal transport (Brandt and Apkarian, 1992; Rajakumar *et al.*, 1993). However, the ability of BDA (3000 MW) to also undergo anterograde transport limits the usefulness of this tracer in multitracing experiments, and the tracer can also be taken up by fibers of passage (Reiner *et al.*, 2000; Veenman *et al.*, 1992).

With regard to the need to visualize the distal dendrites of labeled cells, a method has been introduced for improving axonal uptake of BDA by first applying NMDA *N*-methyl-D-aspartate to lesion the soma and dendrites at the injection site that might compete for uptake of BDA (Jiang *et al.*, 1993). However, in our experience, this procedure does not work for all pathways. For example, when we attempted to use BDA as a retrograde tracer in the PFC to VTA pathway, the prior injection of NMDA into the VTA resulted

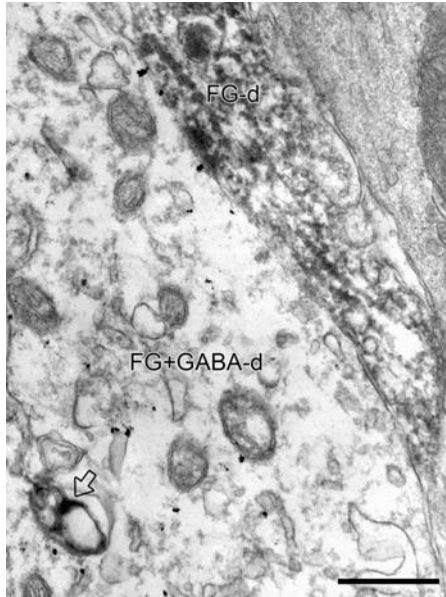
in BDA diffusion throughout a considerable portion of the midbrain with minimal retrograde transport (unpublished data).

## **b. FluoroGold (FG)**

FG has been utilized for some time as an effective retrograde tracer for LM studies (Schmued and Fallon, 1986; Skirboll *et al.*, 1989). More recently, FG has also been adapted as a highly effective tracer for TEM (Chang *et al.*, 1990; Naumann *et al.*, 2000; Van Bockstaele *et al.*, 1994). FG can be delivered as a 2% solution in 0.1 M cacodylate buffer, pH 7.5, by iontophoretic or pressure injections. FG is a highly effective and sensitive retrograde tracer that, to our knowledge, has never failed to label any particular cell population. Neither does this tracer diffuse out of retrogradely labeled cells into adjacent neurons (Novikova *et al.*, 1997; Schmued and Fallon, 1986; Van Bockstaele *et al.*, 1994). Moreover, FG appears to be an exclusively retrograde tracer, at least in the systems for which we have used it to date. Following forebrain or midbrain injections of FG, we have systematically examined sections in the midbrain or pons by TEM and found no evidence for anterograde transport of FG into axon terminals, despite the known existence of available pathways for such transport. The inherent fluorescence of FG also provides a nice benefit to this method, in that the appropriateness of the injection sites and transport can be checked immediately after the brain is sectioned. Misplaced injections or cases of poor transport can then be discarded without loss of more costly reagents.

At the TEM level, FG is seen as being diffusely distributed within the cytoplasm of retrogradely labeled cells or concentrated within lysosomes (Fig. 2.5). In certain neurons, the passage of FG into distal dendrites is excellent, particularly for cells with modest dendritic branching such as monoamine neurons (Carr and Sesack, 2000b; Van Bockstaele and Pickel, 1995). Within these populations, FG appears to undergo equivalent penetration into the distal dendrites of cells that have different axonal targets but share similar dendritic morphology. For example, we have analyzed random ultrastructural samples of dopamine (DA) neurons in the VTA that were retrogradely labeled with FG from the NAc or from the PFC and found no difference in the mean or distribution of the cross-sectional diameters of the dendrites containing FG (unpublished observations). This suggests that FG was transported to an equivalent extent into large and small caliber dendrites in the two populations.

Unfortunately, FG fails to be transported into the distal most dendrites of cell classes with complex dendritic trees, in particular, those with extensive dendritic spines (e.g., cortical pyramidal cells). This difficulty is compounded by the need for immunocytochemistry to detect FG for TEM studies (Chang *et al.*, 1990), with the reduced antibody penetration that accompanies the requirement for ultrastructural preservation. To some extent, this limitation is mitigated by the fact that FG induces the formation

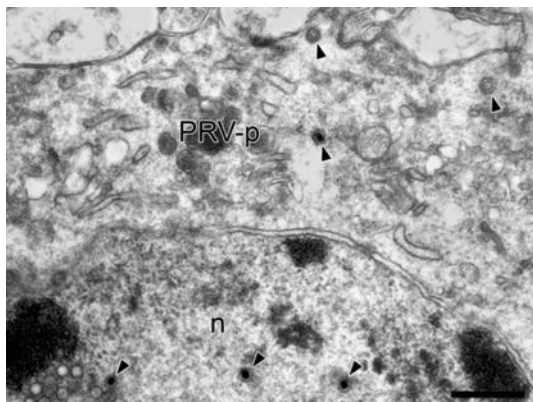


**Figure 2.5.** Electron micrograph of the rat VTA showing dendrites containing immunoperoxidase for FG (FG-d) retrogradely transported from the NAc. The labeling is diffusely distributed to the cytoplasm in one case, but is concentrated in a lysosome (open arrow) in the other. The latter dendrite is dually labeled by immunogold—silver for GABA (FG + GABA-d). Scale bar represents 0.5  $\mu\text{m}$ .

of lysosomes in labeled cells, which can aid in the detection of this tracer (Schmued *et al.*, 1989). However, labeled lysosomes do not always appear in a particular plane of section. Moreover, individual dendrites can exhibit markedly different densities of FG content (Figs. 2.5 and 2.14), with the lowest densities falling near the limit of detection. Another disadvantage of FG is that it can be taken up by fibers of passage (Dado *et al.*, 1990). The iontophoretic application method reduces this drawback (Pieribone and Aston-Jones, 1988), and in our experience, this problem is less with FG than with other retrograde tracers such as cholera toxin (see also Llewellyn-Smith *et al.*, 1990). Nevertheless, it is an important limitation that must be taken into account when interpreting the results of studies using FG. Finally, FG has only limited usefulness for long-term studies. After 1 week, the intensity of labeling begins to diminish progressively (Novikova *et al.*, 1997), and FG can be cytotoxic in some cases (Garrett *et al.*, 1991; Naumann *et al.*, 2000).

### c. Pseudorabies Virus (PRV)

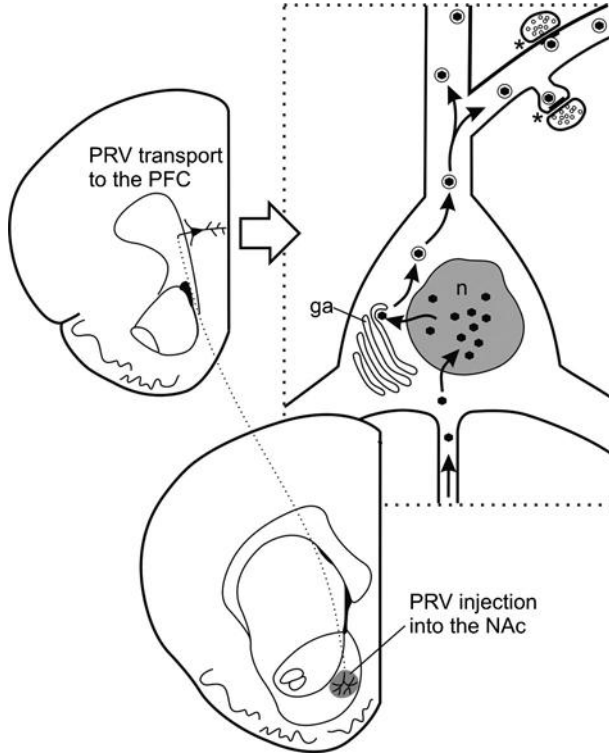
The use of live viruses that replicate within neurons (Fig. 2.6) is an essential tool for retrograde tracing of multisynaptic connections in the nervous system, as detailed in the chapter by Geerling *et al.* and as previously



**Figure 2.6.** Electron micrograph of the rat PFC showing a neuronal perikaryon that is infected with PRV (PRV-p) following transport of this tracer from the NAc. Numerous viral particles (arrowheads) are evident in the cytoplasm and nucleus (n). Scale bar represents 0.5  $\mu\text{m}$ .

described in reviews and the primary literature (Aston-Jones and Card, 2000; Card and Enquist, 1994; Card *et al.*, 1993; McLean *et al.*, 1989; Strick and Card, 1992). In collaboration with J. Patrick Card, we have adapted this technique to label the distal dendrites of neurons infected with virus through a first-order process (Fig. 2.7) in order to study identified synaptic inputs to these dendrites in dual-labeling TEM studies (Figs. 2.8–2.10) (Carr *et al.*, 1999; Carr and Sesack, 2000a). The technique utilizes an attenuated Bartha strain of PRV and has a number of advantages for retrograde tract-tracing.

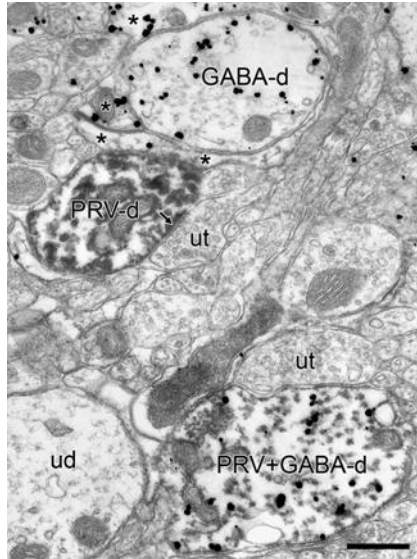
PRV is an exclusively retrograde tracer, although viral strains that undergo anterograde transport are also available (Aston-Jones and Card, 2000). PRV has a high affinity for brain proteins and is rapidly sequestered by neurons at intraparenchymal injection sites, which are typically rather small. The latter may be disadvantageous if large injections are needed to fill a region of interest. Uptake of PRV by fibers passing through the injection site is lower than typically observed with most retrograde tracers (Aston-Jones and Card, 2000; Chen *et al.*, 1999), although such transport can occur and must be taken into consideration for each study. Viral transport is the only tract-tracing method in which the sensitivity is not limited by the amount of tracer incorporated into cells at the time of injection. The ability of the virus to replicate progeny (Fig. 2.6), to traffic viral particles into the dendritic tree (Fig. 2.7), and to cause infected neurons to synthesize viral-specific proteins that concentrate in the soma and dendrites (Figs. 2.8–2.10) provides a degree of signal amplification that is Golgi-like in nature and unique among tracers (Card *et al.*, 1990). In essence, these processes are driven by the necessity for the virus to repeat its life cycle in newly infected cells. Hence, replicated virus is trafficked into the dendrites to facilitate transneuronal passage at sites of synaptic afferent contact (Aston-Jones and Card, 2000; Card *et al.*, 1993). Reactive astrocytes that are drawn to infected neurons take up any



**Figure 2.7.** Schematic diagram illustrating the application of PRV for first-order retrograde transport that labels the distal dendrites of neurons with complex dendritic trees. PRV is taken up by axon terminals at the site of intraparenchymal injection (the NAc in this example) and transported retrogradely (to the PFC in this case). The virus then enters the nucleus (n), in which the virus replicates itself and manufactures viral-specific proteins. Viral particles then acquire two membrane coats from the Golgi apparatus (ga; only one coat is shown here) and then travel into the dendritic tree seeking synaptic sites (\*) at which to pass out of the cell to infect other neurons.

infectious particles that are not transported into axon terminals, and thereby limit nonsynaptic spread. The astrocytes themselves are unable to replicate infectious virus (Aston-Jones and Card, 2000; Card *et al.*, 1993).

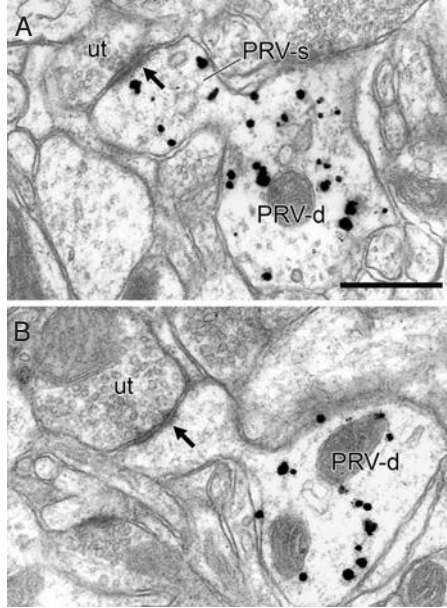
For retrograde tracing in cells with simple dendritic branching patterns, like VTA neurons, we have shown (Carr and Sesack, 2000a) that the ability of PRV to label distal dendrites (Fig. 2.8) is comparable to FG (Fig. 2.5). However, for the identification of the thin distal dendrites and dendritic spines of cortical pyramidal neurons, infection with PRV (Figs. 2.9 and 2.10) is superior to any other tracing method in our experience (Carr *et al.*, 1999; McLean *et al.*, 1989). Moreover, the PRV method is readily amenable to dual-labeling studies of the synaptic inputs to these dendrites (Fig. 2.10) (Carr *et al.*, 1999). Although detection of PRV is based on immunocytochemistry,



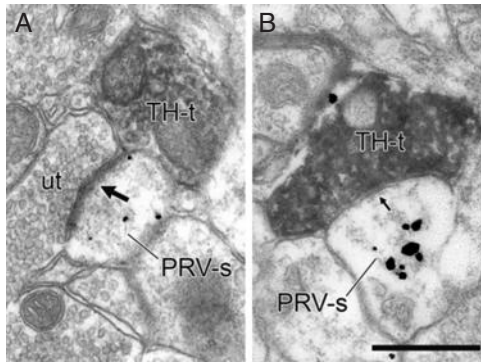
**Figure 2.8.** Electron micrograph of the rat VTA showing distal dendrites that are singly labeled by immunoperoxidase for the retrograde tracer PRV (PRV-d) transported from the NAc, singly labeled by immunogold–silver for GABA (GABA-d), or dually labeled for both markers (PRV + GABA-d). An unlabeled dendrite (ud) is shown for comparison. The PRV-d receives a symmetric synapse (small arrow) from an unlabeled terminal (ut). A second unlabeled terminal is apposed to the PRV + GABA-d without synapsing in this section. Immunogold–silver labeling for GABA is also evident in glial processes (\*). Scale bar represents 0.5  $\mu\text{m}$ .

the primary antibodies are directed against multiple epitopes of viral proteins and so are quite sensitive, even for TEM studies.

Despite the decided advantages of retrograde tract-tracing with PRV for TEM studies of synaptic connectivity, there are substantial limitations that are important to note. It is sometimes difficult to precisely localize PRV injection sites, due to rapid sequestration and transport of the virus (Aston-Jones and Card, 2000). Viruses cause progressive infections that eventually kill the subjects. However, the survival times that are used for most tracing experiments are typically earlier than this period (Kelly and Strick, 2000), and the application of viral tracing for TEM requires only short times needed for first-order infection. Viruses are living elements and so can have considerable differences in their neuroinvasiveness that reflect growth methods, storage conditions, titer, and other factors (Aston-Jones and Card, 2000; Card *et al.*, 1991, 1999). Another significant limitation to viral tracing is the need for biosafety containment facilities in which to inject and house animals. PRV is a swine pathogen that is not infectious to humans, but infected rodents need to be kept isolated from other animals (Strick and Card, 1992). Herpes simplex virus type 1 and rabies virus are infectious to humans and therefore require even more extensive biosafety precautions and facilities



**Figure 2.9.** Electron micrograph of the rat PFC showing immunogold-silver for PRV in spines (PRV-s) and distal dendrites (PRV-d) of pyramidal neurons retrogradely labeled from the contralateral PFC. Immunoreactivity for PRV extends from the parent dendrite into the spine in (A) but not in (B). Both spines receive asymmetric synapses from unlabeled terminals (ut). Scale bar represents 0.5 μm.



**Figure 2.10.** Electron micrographs of the rat PFC showing immunogold-silver for PRV in the spines of pyramidal neurons (PRV-s) retrogradely labeled from the contralateral PFC and immunoperoxidase labeling for tyrosine hydroxylase (TH) in axon terminals (TH-t). (A). The PRV-s receives an asymmetric synapse (large arrow) from an unlabeled terminal (ut) and is apposed by a TH-t that does not form a synapse in this section. (B). The TH-t forms a symmetric synapse (small arrow) onto the PRV-s. Scale bar represents 0.5 μm.

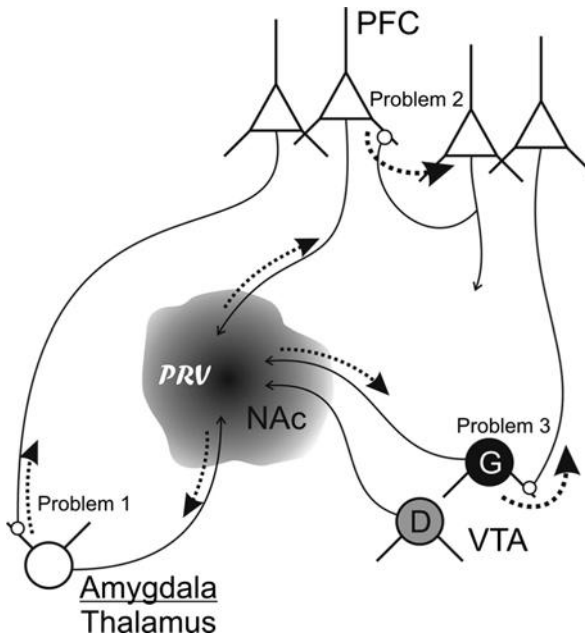
that may be prohibitive for some laboratories (Kelly and Strick, 2000; Strick and Card, 1992).

One of the most attractive aspects of viral tracing, namely its transneuronal transport, may lead to false-positive results when this method is used for TEM studies. Avoiding such outcomes requires careful attention to survival times (Aston-Jones and Card, 2000) and control experiments to ensure that second-order infection has not occurred within the region of interest at the time when TEM studies are performed (Carr *et al.*, 1999). For example, in our prior analysis of cortical pyramidal neurons infected by first-order transport, we monitored second-order infection of GABA local circuit neurons whose synapses onto the pyramidal cells are proximal and therefore likely to be the earliest to pass virus. Survival times at which substantial infection of GABA cells was detected were not used in the TEM analysis of synaptic inputs to pyramidal neurons.

Another notable drawback to PRV tract-tracing is the fact that some neurons may not label with this virus. For example, our repeated attempts to label the known projection from the PFC to the VTA with this method were unsuccessful. The most probable explanation is competition for uptake of the virus at the injection site (Card *et al.*, 1999), with afferents having the largest terminal density being most likely to transport quantities of virus sufficient to mount an infection. Indeed, our injections of PRV into the VTA lead to extensive retrograde transport into brainstem sites, seemingly at the expense of forebrain areas.

As a final consideration of the viral tracing method for use in studying synaptic connectivity, it is worth considering why we have emphasized TEM investigation of first-order transport rather than the better known and more easily applied method of LM analysis of transsynaptic transport. For certain experiments, we agree that carefully timed LM studies of viral trafficking are sufficient to provide evidence of synaptic connections between given cell populations. However, for other circuits, there may be multiple pathways that can transport virus, making it difficult without further manipulations to be certain which course viruses took in moving between synaptically connected cells (Fig. 2.11). For example, if investigating whether the PFC synapses onto DA neurons in the VTA that project to the NAc, one might inject PRV into the NAc and wait for second-order transport into the PFC by way of the VTA. However, following NAc injections, the labeling of PFC neurons could arise from connections within the basolateral amygdala or the paraventricular thalamus rather than the VTA (Problem 1). Indeed, our inability to label the PFC by first-order viral uptake from the VTA (see above) suggests that these alternate pathways would be the more probable routes for virus to reach the PFC. Another likely circuit would involve transneuronal transport of virus into PFC pyramidal neurons via intrinsic synapses onto neighboring pyramidal cells that underwent first-order infection from the NAc (Problem 2). Finally, and perhaps most importantly, even if selective lesions could confine the virus into passing transsynaptically via the VTA, the method would not reveal the phenotype of VTA cells involved in this





**Figure 2.11.** Schematic diagram illustrating the use of PRV for multisynaptic tract-tracing to test whether PFC neurons synapse onto DA cells (D) in the VTA that project to the NAC. Interpretational problems arise (see section “Retrograde Tract-Tracing”) when the virus passes transneuronally through alternate pathways or via GABA cells (G).

transneuronal passage. For example, second-order infection of PFC neurons would most likely occur due to uptake by mesoaccumbens GABA neurons that receive PFC synaptic input (Problem 3) (Carr and Sesack, 2000a). Recent technological developments are beginning to solve the latter problem, for example in creating a form of PRV whose replication is dependent on Cre-mediated recombinant events that can be isolated to neurons with specific phenotype (DeFalco *et al.*, 2001). However, if such an approach is not available for the complex circuit being investigated, the use of TEM to analyze first-order retrograde transport of virus remains a viable option for examining synaptic inputs to identified cell populations.

### 3. Identification of Neurochemical Phenotype

For tract-tracing experiments conducted for TEM analysis, it is often important to identify the neurochemical phenotype of the neurons/dendrites targeted by the traced pathway. For example, we wished to determine the extent to which projections from the PFC innervate DA or GABA neurons in the VTA (Carr and Sesack, 2000a; Sesack and Pickel, 1992). For such studies, antibodies directed against transmitters, enzymes, receptors, transporters,

calcium-binding proteins, or peptides that are unique identifiers of the target cells can be used. Combinations of immunocytochemical markers are then needed to label both the tracer and the phenotypic marker. As described above in section “Dual-Labeling Procedures,” the most typical combination we use is preembedding immunoperoxidase and immunogold–silver, although other combinations are also available. It is important to consider that the peroxidase method is more sensitive than gold–silver by approximately one order of magnitude (Chan *et al.*, 1990). Hence, we typically use peroxidase or immunoperoxidase to label the least abundant antigen (e.g., BDA or PHA-L) and immunogold–silver to label the more plentiful antigen (e.g., TH or GABA).

In other cases, it may be desirable to identify the neurochemical phenotype of neurons containing retrograde tracer from a known injection site. Often such studies are conducted at the LM level, taking advantage of the many available fluorescent markers for double and triple labeling (Skirboll *et al.*, 1989) (see also the chapter by Wouterlood). However, some phenotypes are difficult to detect by LM because a particular marker is not present in sufficient quantity to clearly define the soma. In our experience, this has been a particular problem with GABA in projection neurons. Although the levels of GABA are high in local circuit neurons, they are markedly less robust in projection neurons, such as those in midbrain and basal ganglia structures. Often it is difficult to define these cells using LM methods. Use of GAD antibodies can help with an LM definition of this population, although even this approach requires colchicine treatment to boost GAD levels, and colchicine can interfere with transport of retrograde tracer (Ford *et al.*, 1995). Hence, TEM becomes a useful alternative for determining the extent to which GABA cells contribute to particular projections, as GABA levels are sufficient to detect by TEM. For this purpose, we have followed the protocol originated by Van Bockstaele (Van Bockstaele *et al.*, 1994; Van Bockstaele and Pickel, 1995) to demonstrate the strength of the GABA projection from the VTA to the PFC (Carr and Sesack, 2000b).

Finally, some experiments may call for the identification of neurochemical phenotype in axons that innervate particular brain regions as determined by anterograde tract-tracing. In our experience, this is a particularly difficult issue to resolve using dual-labeling preembedding methods. Although evidence of dually labeled axons is often obtained, it appears that the incidence of such antigen coexpression is underestimated by the combination of immunoperoxidase and immunogold–silver that our laboratory typically uses. This issue is further discussed below in section “Limitations: Sources of False-Negative Errors”.

#### 4. Triple-Labeling Studies

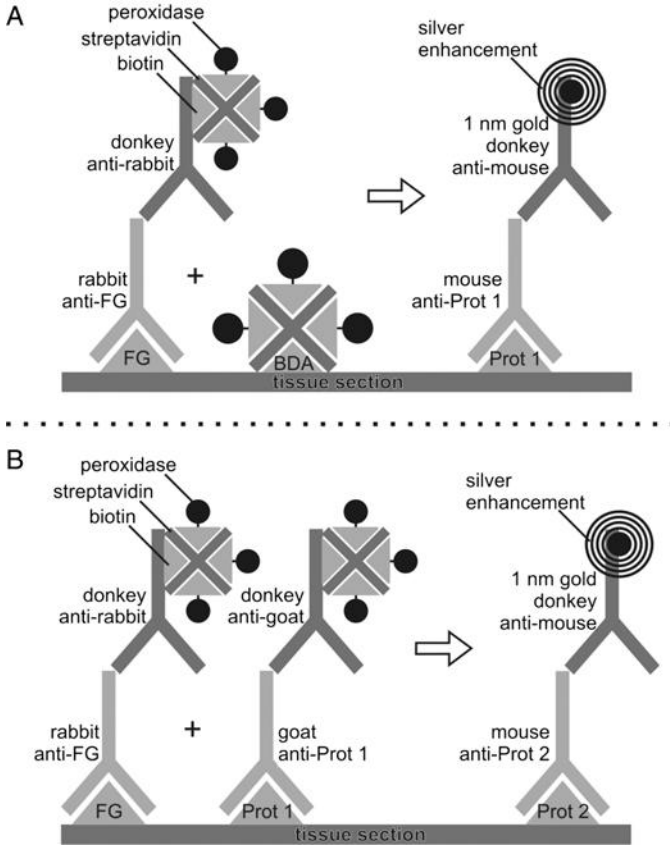
TEM studies can be designed that analyze several combinations of tract-tracing agents and neurochemical markers. The general methodological

principles for combining tracing with neurotransmitter phenotype identification for LM and TEM have been described in a previous volume of this series (Zaborszky and Heimer, 1989). Here, we deal specifically with experiments in which investigators seek to define the synaptic target of a particular anterogradely labeled pathway both in terms of its neurochemical phenotype and in terms of its own projection targets. This requires the combination of two tracers, one anterograde and one retrograde, with a phenotypic marker.

As an example of such an application, we will refer to our findings with regard to the afferent regulation of midbrain DA neurons. The major inputs and outputs of the substantia nigra (SN) and VTA have been known for some time (Oades and Halliday, 1987; Phillipson, 1979; Swanson, 1982), although more minor projections are still the subject of investigation. It is known that the major forebrain projections of the VTA arise from separate populations of neurons, both DA and non-DA, presumably GABA cells (Carr and Sesack, 2000b; Swanson, 1982; Van Bockstaele and Pickel, 1995). DA cells innervating the NAc regulate locomotion and motivated behaviors, whereas DA neurons projecting to the PFC modulate cognitive and affective functions. To understand the morphological basis of behavioral control of DA cell activity, it is important to develop a detailed picture of the specific afferents that synapse onto different populations of VTA DA neurons. Afferents can be identified by anterograde tract-tracing or, in some cases, phenotypic markers, if these derive from single sources (e.g., acetylcholine). Cell populations can be defined on the basis of retrograde tract-tracing from known target areas (e.g., PFC and NAc), and neurotransmitter identity of these populations can be delineated based on immunocytochemistry for transmitter markers. In the first study of its kind, our laboratory used a combination of anterograde and retrograde tract-tracing with immunocytochemistry to show that excitatory projections from the PFC synapse selectively onto DA and not GABA neurons that project back to the PFC and onto GABA but not DA cells that innervate the NAc (Carr and Sesack, 2000a). Such synaptic specificity may explain some of the unique functional properties of mesoprefrontal and mesoaccumbens neurons and help to advance understanding of the circumstances and mechanisms for their behavioral activation.

More recently, we have performed a similar study of the inputs to the VTA from the brainstem laterodorsal tegmentum (LDT), which, unlike the PFC, synapses onto DA neurons that innervate the NAc (Omelchenko and Sesack, 2005a). The LDT projection contains a mixed neurochemical phenotype, and with this in mind, we have also completed an analysis of cholinergic inputs to different VTA cell populations (Omelchenko and Sesack, 2005b), as the cholinergic innervation derives predominantly from the LDT (Hallanger and Wainer, 1988; Oakman *et al.*, 1995).

The success of these experiments depends on the combination of immunoperoxidase and immunogold–silver labeling methods, and on the use of one of these markers, typically immunoperoxidase, to label two of the three desired components that are known to be segregated in different

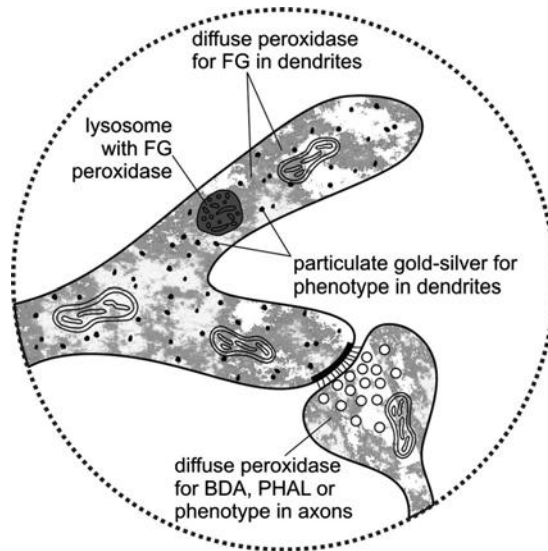


**Figure 2.12.** Schematic diagrams showing the labeling procedures used for triple labeling in the experiments described here. (A) Two primary antibodies raised in different species against FG and phenotypic protein 1 (Prot 1) are co-applied, and then a biotinylated secondary antibody against the first species is added, followed by the avidin–biotin peroxidase complex. The latter will also bind to the biotin in the anterograde tracer BDA. Following peroxidase histochemistry, a gold-conjugated secondary antibody against the second species is added, and the bound gold particles are silver enhanced. (B) Three primary antibodies raised in different species against FG, a protein labeling the afferent pathway (Prot 1; e.g., PHA-L or a unique phenotypic marker such as VAcHT) and a phenotypic protein labeling the target (Prot 2) are co-applied. A mixture of biotinylated secondary antibodies against the first two species is then applied, followed by the avidin–biotin peroxidase complex. Following peroxidase histochemistry, a gold-conjugated secondary antibody against the third species is added, and the bound gold particles are silver enhanced.

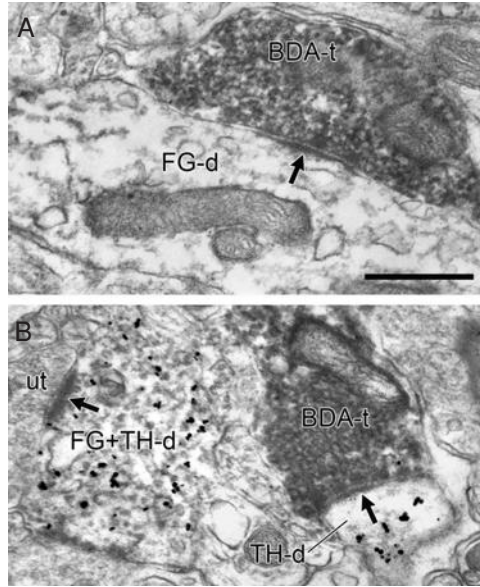
neuronal compartments. In the most typical case, BDA is used as the anterograde tracer, and FG is the retrograde tracer of choice. Immunoperoxidase labeling for FG in soma and dendrites is accomplished using the ABC method, which will by design also label the axons that contain BDA (Fig. 2.12A). Immunogold–silver is then used to label the phenotypic marker

in the soma and dendrites. In this case, the primary antibodies against FG and the phenotypic protein must be raised in separate species. The method can also be adapted for the use of PHA-L as the anterograde tracer or the use of a phenotypic marker that labels a class of afferent axons from a known source, for example the vesicular acetylcholine transporter (VAcHT) to label cholinergic afferents to the VTA that are known to derive from the brain-stem tegmentum. In this case, there are three primary antibodies, and each must either be from a different species (Fig. 2.12B) or two can be from the same species as long as they will be segregated into different compartments (e.g., rabbit anti-FG and rabbit anti-PHA-L).

It is important for these experiments that appropriate controls are run to verify the segregation of immunoperoxidase markers. Sections processed only for FG should reveal peroxidase product for this tracer only in dendrites, either diffused in the cytoplasm or concentrated in lysosomes (Figs. 2.5 and 2.13), and not in axons. Similarly, sections processed only for the anterograde tracer should reveal peroxidase product for this tracer diffusely distributed within axons and not soma or dendrites (Fig. 2.13).



**Figure 2.13.** Schematic diagram illustrating the differential distribution of tracers and phenotypic markers within different compartments in an experiment in which anterograde and retrograde tract-tracing are combined. Peroxidase or immunoperoxidase is used to label both the retrograde tracer FG within soma and dendrites and the anterograde tracer BDA or PHA-L (or a unique phenotypic marker) within axon terminals. Control experiments are needed for each system under study to ensure that the tracers label only their respective compartments. Immunoperoxidase is typically diffusely distributed within these compartments but is occasionally concentrated within lysosomes in the case of FG. Finally, preembedding immunogold–silver is used to label antigens unique for different neuronal phenotypes in the retrogradely labeled cell population(s).

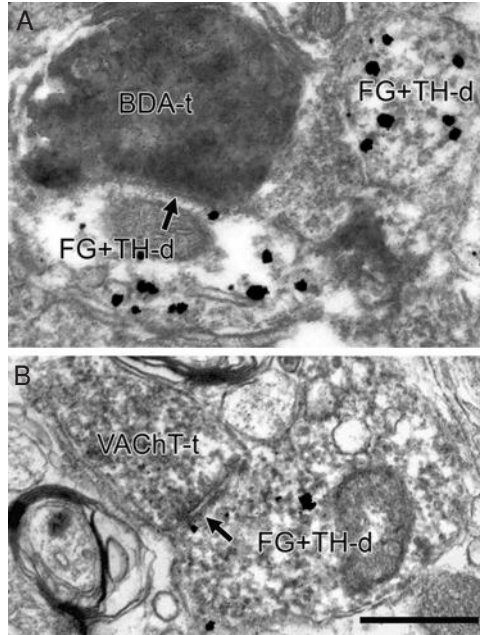


**Figure 2.14.** Electron micrographs of the rat VTA showing the juxtaposition of axon terminals labeled by peroxidase for BDA (BDA-t) anterogradely transported from the LDT, dendrites singly labeled by immunoperoxidase for FG (FG-d) retrogradely transported from the NAc, singly labeled by immunogold–silver for TH (TH-d), or dually labeled for both markers (FG + TH-d). (A) The BDA-t forms an asymmetric synapse (large arrow) onto the FG-d. In (B) the BDA-t synapses onto the TH-d, while the FG + TH-d receives synaptic input from an unlabeled terminal (ut). Scale bar represents 0.5  $\mu\text{m}$ .

This is especially important for BDA, which is capable of some degree of retrograde transport in certain neuronal pathways. The segregation of the immunogold–silver is less of a concern, as it may appear in soma and dendrites (Fig. 2.13) as well as axons, for example in the case of GABA.

Using this approach, it is possible to see synaptic contacts on several different dendrite populations. For example, the anterograde tracer may occur within axon terminals that synapse onto dendrites containing only the retrograde tracer and not the phenotypic marker (Fig. 2.14A) or onto dendrites that are phenotypically labeled but do not contain the retrograde tracer (Fig. 2.14B). However, in fortuitous cases, the axons containing anterograde tracer are observed to synapse onto dendrites that contain both the retrograde tracer and the phenotypic marker (Fig. 2.15A). Alternatively, axons labeled by a phenotypic marker (e.g., VAcHT) will synapse onto dendrites that have these characteristics (Fig. 2.15B).

For this method to provide a useful estimate of the extent to which synapses occur between identified inputs and outputs of a region, it is necessary to perform a certain degree of analysis in serial sections. In many cases, labeled axons may be closely apposed to labeled dendrites without synapsing



**Figure 2.15.** Electron micrographs of the rat VTA showing axon terminals labeled by peroxidase for BDA (BDA-t in A) anterogradely transported from the LDT or for VACHT (VACHT-t in B) forming asymmetric synapses (large arrows) onto dendrites dually labeled by immunoperoxidase for FG retrogradely transported from the PFC or the NAc, respectively, and immunogold–silver for TH (FG + TH-d). In (A) a second FG + TH-d does not receive synaptic input in this section. Scale bar represents 0.5  $\mu$ m.

onto them in one plane of section. In this case, synapses may be revealed in immediately adjacent sections. Serial sections also assist the determination of whether low levels of gold–silver particles are repeated over the same structure in adjacent sections and therefore likely to be specific. However, in this case, it would be preferable to examine sections closer to rather than further from the surface (see Fig. 2.16). In order to thoroughly investigate the possible presence of synapses onto each identified cell population, it is also necessary to sample extensive amounts of tissue. This is particularly true if the investigator uses mesh grids as recommended in section “General Sampling Issues,” as the metal obscures part of the tissue being examined. As further discussed in that section below, extensive sampling is necessary to address the possibility that failure to find a particular synapse type is not due simply to underrepresentation in the sample.

Other investigators have developed a triple-labeling approach that utilizes two different anterograde tract-tracing agents and examines whether both inputs converge onto a common target neuron (French and Totterdell, 2002, 2003). In this case, the target neuron is defined not by neurochemical phenotype but by morphological type, as assessed by its uptake of locally

administered BDA. The success of this method relies on careful comparative analyses between LM and TEM in order to identify different qualities of immunoperoxidase for the two anterograde tracers and identify regions of probable synaptic input to the target neuron. These regions are then analyzed by TEM to verify the presence of synapses.

### III. PRINCIPLES OF THE METHODS

Although tract-tracing and immunocytochemical methods can be combined in a variety of ways, the major steps of the TEM preembedding procedures are usually the same. First, the animal is deeply anesthetized until all pain reflexes are gone. The animal then undergoes transcatheter perfusion with aldehydes to fix proteins. The brain blocks are sliced with a Vibratome; some method is used to enhance antibody penetration; and free-floating brain sections are incubated in antibody solutions. Tissue lipids are then fixed with osmium tetroxide, and sections are dehydrated and embedded in plastic resin. After ultrathin sectioning and counterstaining, the tissue is then examined by a TEM. Many of these procedures are common to LM immunolabeling and so involve similar technical concerns: antibody specificity, prevention of nonspecific labeling, penetration enhancement, etc. However, it is important to appreciate that TEM studies are not simply LM experiments with some extra steps. The experiments must be designed with TEM in mind, which typically involves altering procedures in order to balance maximal preservation of ultrastructure with optimal detection of the desired antigens.

#### A. Animals

The methods presented here are designed primarily for small rodents, although they can be adapted for larger animals. Moreover, many of the immunocytochemical and sampling procedures are fully applicable to other species once fixation is completed and brain sections are cut. An important question to consider for TEM studies is how many animals are typically needed. The answer varies depending on the details of the experiment. If tract-tracing is part of the design, additional animals are often required to allow for misplaced injections. If a new antibody is being tested, pilot studies of optimal fixatives and dilutions are needed. Once all the parameters are optimized, the number of animals required may depend on the results that are obtained. Abundant proteins or highly robust synaptic connections will typically be observed in multiple samples from within and across animals. In that case, three animals each showing similar protein localization or a similar frequency of synapse detection may be sufficient. The more the data seem to vary within or between animals, the greater the sampling required to ensure that the conclusion being developed accurately represents reality.



A question that relates to the issue of variability is the extent to which animals with different densities of labeling should be represented in the overall sample. Obviously, one would not be expected to produce a large sample from an animal in which the immunodetection procedures were clearly suboptimal, just to say that animals were sampled equivalently. Such an approach is sure to produce false-negative results. Conversely, if the tissue from one particular animal seems to have been blessed with the most optimal immunolabeling and ultrastructural preservation, it is tempting to overrepresent this animal in the sample, arguing that it is actually more representative of reality. Of course, a balance needs to be struck between the desire for absolute rigor and the search for “truth” in neuroanatomy. Animals with poor immunolabeling should be excluded from quantitative analyses, and conclusions should not be based on data from single animals. Moreover, clearly stating in published work how the sample was collected and how extensively each animal was represented in the sample should allow colleagues to make their own conclusions regarding the validity of the findings.

### **B. Tract-Tracing**

The principles of experimental design for combining anterograde and retrograde tract-tracing with immunochemical markers have been extensively described in the sections above. Here, we wish to present some methodological issues associated with animal welfare. The surgery necessary to introduce multiple tract-tracing agents can be rather long. However, we chose to combine tracers that have similar survival times (e.g., BDA and FG) specifically so that they can be injected during a single survival surgery. Most institutions require extensive justification for performing multiple survival surgeries, and for the best interests of the animals, we endeavor to avoid such designs. Investigators must choose an anesthetic regimen that can be maintained over several hours. We prefer to use a mixture of ketamine (34 mg/kg), xylazine (7 mg/kg), and acepromazine (1 mg/kg) that is injected i.m. For long surgeries, it is also imperative that the animals' temperature be monitored with a rectal probe and maintained at 36.5°C using a thermostatically regulated heating pad. It is also important to monitor animals closely during recovery from surgery and to administer analgesic if they show any signs of pain or discomfort. We recommend butorphanol, 2 mg/kg, s.c. every 8 h as needed.

### **C. Phenotypic Labeling**

In the sections above, we have also extensively described the principles of immunocytochemistry as related to detection of neurochemical phenotype. One issue that was not addressed is the use of intracerebral (typically intraventricular) injections of colchicine to enhance the content of certain proteins or peptides so that they may be more readily detected by LM or TEM (Dube and Pelletier, 1979; Ford *et al.*, 1995; Graybiel and Chesselet, 1984;

Ribak *et al.*, 1978). By disrupting axonal transport (Paulson and McClure, 1975), colchicine leads to accumulation of peptide in soma and sometimes in dendrites, thereby improving the sensitivity of antigen detection. Most commonly, colchicine is injected into the lateral ventricles and allowed to perfuse the brain through the ventricular system. However, local injections of colchicine within a brain region can reveal the most likely sources of peptide-containing afferents that can then be verified with retrograde tract-tracing (Arluison *et al.*, 1994). In either case, colchicine interferes with axonal transport of tracing agents (Monti-Graziadei and Berkley, 1991), and so if the goal of an experiment is to examine phenotypic markers in retrogradely labeled cells, it is necessary to inject the tracer first and perform intracranial injection of colchicine in a second survival surgery. Because colchicine treatment is distressful for animals, it is recommended that the second survival time be no more than 24–48 h and that butorphanol is given at 2 mg/kg, s.c. every 8 h to relieve suffering. It should also be borne in mind that the full consequences of colchicine treatment are not yet understood. Data indicate that the drug may evoke abnormal gene expression, protein synthesis, and morphological changes within neurons (Pirnik *et al.*, 2003; Rho and Swanson, 1989; Yan and Ribak, 1999).

## D. Intracardial Perfusion

### 1. Pretreatments

Certain neuronal elements contain endogenous metals (e.g., zinc in glutamate nerve terminals) that can complex with silver during the silver enhancement steps for preembedding immunogold–silver labeling. Hence, animals are first treated with a zinc chelator to minimize this source of spurious labeling (Veznedaroglu and Milner, 1992).

### 2. Choice of Fixative

Immunocytochemical staining methods necessitate labeling antigens in fixed cellular material. The goal of tissue fixation for immunocytochemical processing is the preservation of tissue in a state as close to natural as possible while maintaining the ability of the antigen to react with the antibody. Rapid, thorough preservation of the brain is required. A detailed description of the fixation procedures used in our laboratory is provided in the Appendix. However, it should be noted that the characteristics of some antibodies allow optimal labeling only following certain types of fixation. For instance, the antibody against the neurotransmitter DA is generated against a peptide sequence that is conjugated to glutaraldehyde (Chagnaud *et al.*, 1987). Because of this trait, labeling with this antibody is feasible only following fixation using high amounts of glutaraldehyde. On the other hand, certain fixatives may denature antigens, resulting in little to no staining.

Therefore, the optimal fixative for each antigen–antibody combination must be determined empirically.

The standard fixative for TEM is typically some combination of 4% formaldehyde and glutaraldehyde in concentrations from 0.05 to 1% depending on compatibility with primary antibodies. However, in our experience, certain antibodies actually produce better immunolabeling in tissue fixed with 2% formaldehyde and 3.75% acrolein, a related aldehyde that gives excellent tissue preservation for TEM. This can be true even for antibodies that label only poorly in glutaraldehyde-fixed sections, such as those generated against the monoamine plasma membrane transporters described above. Hence, we consider it worth the effort to attempt acrolein fixation in testing new antibodies, although many laboratories avoid this chemical because the hazardous risks associated with it are considered greater than for glutaraldehyde. The basic procedure for acrolein perfusion was presented in Leranthe and Pickel (1989). Here in the Appendix, we provide information on how to safely mix acrolein solutions and perform intracardial perfusions in rodents without undue risk to personnel. Of course, each laboratory should become familiar with the material safety data sheet for acrolein as for any hazardous chemical.

Regardless of the fixative employed, the speed at which fixation of brain tissue is accomplished is essential for the best ultrastructural preservation. In our experience, the time between the opening of the animal's diaphragm and the introduction of fixative should be as short as possible (20–30 s). Extensive saline rinsing to remove blood cells only delays the time at which fixative is introduced. Hence, we recommend the inclusion of heparin with the initial saline rinse to quickly remove blood cells and prevent clotting within the vasculature. It is also important to avoid introducing air bubbles into the system, as these might also block vessel perfusion.

### **3. Postfixation and Sectioning**

A well-fixed brain should contain no visible blood and be firm to the touch. It should be ready for sectioning on a Vibratome after a short postfixation period (30–60 min). Postfixation is usually performed in the final fixative that was perfused through the animal. Inappropriately fixed tissue will often be difficult to section, and although the brain may be further hardened by postfixation overnight, such prolonged exposure to fixative can render antigens of interest inaccessible to primary antibodies. In our opinion, a brain that does not section well should be abandoned unless it is quite valuable. Cutting on a Vibratome is the recommended method of sectioning brain material, as it avoids any artifacts that would be associated with frozen sectioning. As presented previously in this series (Leranthe and Pickel, 1989), it is recommended that sections be treated with a brief incubation in 1% sodium borohydride in order to stop fixation (by reducing any aldehydes still exposed to the tissue) and reduce background labeling.

Finally, in the event that immunocytochemical labeling cannot proceed immediately, or the investigator wishes to save sets of tissue for future analysis (not unusual in the case of tract-tracing studies), it is possible to store sections in a cryoprotectant solution (see Appendix, section “Cryoprotection and Tissue Storage”). However, pilot studies should be run to determine whether this storage alters antigenicity for any given protein.

## E. Immunocytochemistry

### 1. Penetration Enhancement

For many immunocytochemical experiments, materials or procedures are introduced to the tissue sections to disrupt cellular membranes in order to potentiate antibody penetration, and thus increase the level of labeling. However, because the membranes must remain visibly intact for electron microscopic examination, only the mildest techniques for enhancing antibody penetration can be employed. The typical compounds used for TEM are detergents such as Triton X-100 (0.04%) or surfactants like PhotoFlo (0.1%). Some investigators use a slightly higher concentration of these reagents but only briefly expose tissue to them during the normal serum blocking procedure. Alternatively, the lower concentrations can be used throughout the primary antibody incubation. In our experience, the use of detergents like Triton tends to reduce background nonspecific labeling, which is advantageous. On the other hand, we have found a few proteins whose antigenicity is actually reduced by detergent treatment (Luedtke *et al.*, 1999; Sesack and Snyder, 1995), most likely due to disruption of transmembrane domains upon solubilization. Hence, penetration enhancement can also be accomplished by treating tissue with a cryoprotectant and subjecting it to rapid freezing and thawing using either liquid nitrogen or a  $-80^{\circ}\text{C}$  freezer. The recipe for the latter procedure is given in the Appendix.

### 2. Choice of Markers

TEM involves passing a beam of electrons through the tissue specimen. Therefore, only markers that can trap electrons and thus appear “electron dense” can be visualized with this method. In contrast to the use of varied colors for LM techniques, there are only a few types of markers that can be distinguished from each other at the TEM level. These include autoradiography (i.e., silver grains developed in a photographic emulsion), gold with or without silver enhancement, and peroxidase chromogens. Autoradiographic immunolabeling has been discussed in detail in a previous chapter in this series (Pickel and Milner, 1989). This method has high sensitivity, and the product is easy distinguishable from immunogold and immunoperoxidase markers. However, it can take 3–12 months to develop autoradiographic material for TEM. Moreover, it is not entirely suited for subcellular

localization studies, as radioactivity can spread to produce silver grains a short distance away from antigen sites. In recent years, immunautoradiography has generally been replaced by methods that are quicker to develop and provide more discrete localization.

In the sections above, we have provided general information regarding the advantages and disadvantages of using immunoperoxidase versus immunogold–silver for preembedding studies of protein localization or synaptic connectivity. Here, we discuss more specific details of the procedures within these two general classes.

### **a. Preembedding Immunoperoxidase**

One of the earliest types of immunoperoxidase labeling involved the application of a soluble complex of peroxidase enzyme bound to antiperoxidase antibodies, termed PAP (for peroxidase antiperoxidase) (Sternberger, 1974). This procedure, which has been previously discussed in this series (Pickel, 1981), involves primary antibody binding to tissue antigens, followed by application of a secondary antibody raised in another species and directed against the species in which the primary antibody was raised, and finally addition of the PAP complex for which the antiperoxidase is raised in the same species as the primary antibody. Following exposure of the peroxidase enzyme to a chromogen substrate (e.g., DAB) and  $H_2O_2$ , an electron-dense reaction product is formed. The density of the peroxidase product can then be further enhanced by osmication of the tissue (Johansson and Backman, 1983). Moreover, an additional round of exposure to the secondary antibody and the PAP complex produces amplification of the peroxidase signal (Ordroneau *et al.*, 1981). Subsequent efforts to enhance even further the incorporation of peroxidase, and thus the sensitivity of the method, lead to the introduction of the ABC technique (Hsu *et al.*, 1981). For this procedure, the secondary antibody is conjugated to several molecules of biotin. The tissue is then exposed to a solution containing avidin, which binds to biotin with high affinity, and biotinylated horseradish peroxidase. Similar to the other types of immunoperoxidase procedures, exposure of the enzyme to a chromogen results in a flocculent, electron-dense reaction product. Although most laboratories now use the ABC method as their sole immunoperoxidase technique, we continue to find applications for the PAP method, for example when using immunogold–silver to localize compounds like BDA that would otherwise produce false dual labeling if exposed to ABC (Pinto and Sesack, 1998). In addition, the presence of endogenous biotin in some glial cells (Yagi *et al.*, 2002) may present a circumstance in which the PAP method would be preferred to ABC.

As previously reviewed in this series, there are several chromogens that can be used for immunoperoxidase, the most common being DAB, benzidine dihydrochloride, tetramethylbenzidine, VIP peroxidase, and SG peroxidase (both from Vector Laboratories Burlingame, CA) (Warr *et al.*, 1981;

Zaborszky and Heimer, 1989; Zhou and Grofova, 1995). DAB is the most commonly used chromogen for TEM studies, as it produces the smallest crystalline size and therefore the most diffuse, flocculent reaction product that can fill even small neuronal processes. It is also more stable for the TEM processing steps than tetramethylbenzidine. More recently, VIP peroxidase has shown excellent sensitivity and stability for TEM studies, and its distinctive rosette-like appearance may provide easier recognition within dendrites where the diffuse nature of the DAB reaction product may sometimes be difficult to discern (Zhou and Grofova, 1995). Benzidine dihydrochloride appears to be less sensitive than DAB or VIP peroxidase and its precipitates are not always uniform in appearance (Zhou and Grofova, 1995). Nevertheless, it is still a useful marker for abundant antigens in large processes such as soma and proximal dendrites (Charara *et al.*, 1996). Tetramethylbenzidine is reported to have greater sensitivity than other chromogens, but its instability in aqueous solutions and alcohol requires that it be stabilized for use in TEM (Llewellyn-Smith *et al.*, 1993; Marfurt *et al.*, 1988; Rye *et al.*, 1984). Such stabilization procedures can result in some loss of sensitivity. Because these different immunoperoxidase chromogens produce precipitates of different size, texture, and appearance, they can be used in combination for dual-labeling studies (Norgren and Lehman, 1989; Smith *et al.*, 1994; Zaborszky and Heimer, 1989; Zhou and Grofova, 1995). However, it may not always be possible to distinguish the presence of two different peroxidase products within the same neuronal structure, especially axon terminals (Zhou and Grofova, 1995) (see also section “Limitations: Sources of False-Negative Errors”).

Another important consideration for TEM studies is the strength of the immunoperoxidase reaction. We have experimented with changing the concentration of the DAB chromogen, the concentration of the  $H_2O_2$ , and the duration of the incubation. In our experience, the main determinant of the amount of peroxidase product generated is the incubation time. The time in solution should be chosen empirically: inadequate exposure will not label antigens deep in the tissue, whereas overexposure will obscure ultrastructural detail. The peroxidase product in overstained profiles may also pierce the plasma membrane and spread into adjacent profiles. This is usually readily detectable in the TEM as peroxidase product in the vicinity of broken membranes. In the event that the sensitivity of immunoperoxidase is needed for a study but a clear view of cellular detail is also critical, the DAB precipitate can be intensified by metallic silver grains through an argyrophil III reaction (Gallyas, 1982). This procedure has been applied with high sensitivity in TEM studies (Liposits *et al.*, 1984) and has been further modified to allow enhancement of DAB reaction product that is deliberately produced at a low level so as not to obscure subcellular detail (Smiley and Goldman-Rakic, 1993). A further variant of this approach is to perform silver-gold enhancement of a nickel-DAB precipitate, and this method produces a reaction product that can be distinguished from nonenhanced DAB for dual-labeling TEM studies (Hajszan and Zaborszky, 2002). In this regard, it should be noted that it is not uncommon to find light silver labeling of

immunoperoxidase in tissue that has been dually labeled by the standard ABC and preembedding immunogold–silver methods. However, the size of these particles is generally quite small and therefore readily distinguished from specific immunogold–silver particles.

### **b. Preembedding Immunogold–Silver**

The protocols for immunogold–silver labeling are rather different from the immunoperoxidase steps, and the distinction begins at the time of sacrifice. As noted above, it is recommended that the animal should be treated with a zinc chelator prior to perfusion in order to prevent silver intensification of endogenous zinc (Veznedaroglu and Milner, 1992). Another approach using L-cysteine exposure to reduce tissue argyrophilia in fixed sections has also been developed (Smiley and Goldman-Rakic, 1993).

Following incubation of sections in primary antibody, they are exposed to secondary antibodies conjugated to “ultrasmall” gold particles in the range of 1  $\mu\text{m}$ . There are several commercial sources of these antibodies as well as the silver enhancement solutions that are matched to them. The most common sources of silver reagents used for preembedding immunogold–silver are Amersham Biosciences Corps (Piscataway, NJ), Nanoprobes Inc. (Yaphank, NY), and Aurion (Electron Microscopy Sciences, Fort Washington, PA). Regarding dual- or triple-labeling studies such as those described here (sections “Dual-Labeling Procedures” and “Triple-Labeling Studies”), it is important to note that Electron Microscopy Sciences offers a line of gold-conjugated secondary antibodies raised in donkey. We have found that having all secondary antibodies from donkey helps to avoid species cross-reaction.

A critical determinant for the success of preembedding immunogold–silver is the size of the gold–silver particles, and hence the total incubation time in silver reagents. This issue has been extensively covered in the original reviews of the method (Chan *et al.*, 1990; Pickel *et al.*, 1993). Briefly, at short incubation times, labeled structures appear light gold at the LM level. As silver intensification proceeds, the color moves increasingly toward brown and finally black. In addition, the levels of nonspecific gold–silver deposit increase with time. The LM appearance of specific gold–silver labeling that is considered optimal for TEM is generally in the range of brown. The total incubation time to achieve this is empirical and based largely on the primary antibody and the density of the antigen. It is recommended that each experiment involve a timed series (4–12 min) on a few test sections and then bulk processing of the remaining tissue sections at two different time points, one that is deemed optimal by LM (clear detection of the structures of interest with minimal evident background) and one that is 1–2 min shorter.

The silver processing steps also require extensively clean glassware, which usually involves acid washing. In our experience, it is more convenient,

though admittedly more costly, to use disposable cultured cell well plates for this purpose. In addition, there must be no metal ions in the tissue at the time when the enhancement procedure is performed. Any metal ions present in the tissue will be silver intensified in addition to the gold particles, resulting in nonspecific labeling. Therefore, the tissue must be manipulated using nonmetal instruments. For this purpose, our laboratory employs wooden applicator sticks.

Another important issue is the temperature at which the silver reaction is conducted. The amount of time necessary for the silver enhancement is highly dependent on the temperature of the silver solution (i.e., the warmer the solution, the shorter the silver enhancement time required). In our laboratory, we store the silver solutions in the refrigerator, but allow them to reach room temperature prior to use. Furthermore, once the silver enhancement has been performed, the solutions to which the tissue is subsequently exposed must be at room temperature. In other words, the osmium tetroxide solution must be at an ambient temperature prior to osmication of silver-intensified immunogold-labeled tissue. Otherwise, it is possible that tissue expansion and contraction upon temperature changes will dislodge silver-enhanced gold particles from the tissue.

### **3. Parallel Versus Serial Antibody Incubations**

In our experience, the most efficient way to perform dual or triple immunocytochemical labeling is to incubate sections in primary antibodies raised in different species in a parallel manner, followed by serial applications of secondary antibodies for immunoperoxidase and immunogold–silver respectively. Of course, it is also possible to apply primary antibodies in a sequential manner, performing all of the immunoperoxidase procedures before incubation in the second primary antibody. However, we have observed at least one case in which low levels of peroxidase reaction product were washed out during subsequent lengthy antibody incubations. Hence, we continue to prefer the parallel versus the serial approach.

### **4. Antibody Dilutions**

The optimal dilution of primary antibody will vary considerably and must be empirically determined for each antigen–antibody combination and for each brain region. For example, in the VTA where TH immunoreactivity in soma and dendrites is abundant, anti-TH antibodies can be used at higher dilution than in the forebrain where the levels of TH in axons are lower. Moreover, the concentration of primary antibody often has to be several times higher for immunogold–silver than for immunoperoxidase due to the lower sensitivity of the former method. For initial pilot studies, a range of antibody dilutions should be tested that include concentrations published



in the literature. Additional recommendations for dilution testing have recently been published (Saper, 2003).

## 5. Antibody Controls

It is essential for any laboratory using immunocytochemical methods to establish the specificity of the antibodies they are employing. The appropriate control experiments for immunocytochemical detection of antigens for TEM are similar to those required for LM or for confocal microscopy and have been discussed elsewhere (Saper, 2003). Briefly, lack of immunolabeling must be observed following: (1) omission of the primary antiserum, (2) exclusion of the secondary antiserum, (3) preadsorption of the primary antibody with the antigen prior to exposure to the tissue, and, where possible, (4) incubation of tissue sections from transgenic mice in which the antigen has been “knocked out.”

When performing double- and triple-labeling procedures, special attention should be paid to ensure absence of cross-reaction between secondary antibodies. Obviously, the best results can be achieved if all secondary antibodies used in the study are obtained from the same species. However, the immunological similarity between certain species (e.g., sheep and goat) can still lead to problems, and we have sometimes noted cross-reaction of certain antibodies against immunologically different species (e.g., anti-rabbit IgG labeling rat primary antibodies). For biotinylated IgG, we recommend the use of antibodies with minimal species cross-reaction, for example those obtained from Jackson ImmunoResearch Laboratories, West Grove, PA. However, as gold-conjugated antibodies are unlikely to be developed for minimal species cross-reaction, it is still necessary to perform the following control experiment. Incubate tissue sections in a mixture of primary antibodies that are known to have distinct compartmentalization or are known not to colocalize (e.g., rabbit anti-TH and mouse anti-GABA in striatal sections). Then divide the tissue into two sets and perform dual immunoperoxidase and immunogold–silver with one of the two secondary antibodies left out of each set. Evidence of markers in inappropriate compartments or unexpected dual labeling of structures will indicate that a particular secondary antibody labels more than its respective species.

## F. Tissue Preparation for TEM

### 1. Osmication

Treatment of tissue sections with osmium tetroxide provides fixation of lipids by rendering them insoluble prior to dehydration and plastic embedding. Osmium also imparts a heavy metal stain to the lipids, enhancing the contrast of membranes. However, osmium is a strong oxidizing agent that

can alter the antigenicity of many proteins, making it a challenge to immunocytochemically label such proteins following plastic embedding (Hemming *et al.*, 1983). Consequently, investigators have developed methods to substitute other chemicals for osmium to achieve lipid fixation without loss of antigenicity (Phend *et al.*, 1995).

For preembedding procedures, the greatest challenge with osmium is that it can oxidize silver metal to silver salt with resultant loss of the silver used to enhance immunogold labeling. In our experience, loss of silver during the osmication step is primarily associated with the presence of chloride ions in the buffering solution. The use of PBS (phosphate-buffered saline) or PB buffers that have been pH adjusted with HCl introduces chloride anions that can form a salt with silver cations, and this seems to greatly speed the oxidation of silver metal to silver salt. Indeed, when inadvertently using solutions that contained chloride, we have witnessed the oxidation of silver in brain sections to nondetectable levels within 1–2 min. Conversely, scrupulous avoidance of chloride ions (or other halides that can readily form silver salts) can reduce this problem and slow the rate of oxidation so that it is minimal within the typical times used for osmication. Hence, the buffer mixed with osmium tetroxide should be PB that has been pH adjusted with phosphoric acid and contains no chloride ions. Using this reagent, it should not be necessary to shorten the osmication time.

## 2. Dehydration

Preparation of tissue for electron microscopy requires fixation of lipids followed by extensive dehydration. During these procedures, the volume of tissue decreases (Hillman and Deutsch, 1978), suggesting that a portion of the extracellular space has been lost. Therefore, caution must be exercised in determining some measurements, such as distance between labeled structures, and it should always be acknowledged that such measurements are only semi-quantitative in nature.

## 3. Counterstaining

Tissue to be examined by electron microscopy is typically stained with heavy metals (first osmium and then uranyl and lead) in order to increase the electron density of membranes and enhance contrast. Many procedures call for application of uranyl acetate *en bloc*, meaning on sections during the alcohol dehydration steps (usually with the 70%). However, such treatment can sometimes make it difficult to identify sparse immunoperoxidase labeling, such as that associated with low abundance proteins. For this reason, we routinely apply heavy metal stains only after ultrathin sectioning and only on a portion of the grids. In this case, the investigator has the option to omit uranyl acetate and stain ultrathin sections only with lead citrate in order to visualize low amounts of immunoperoxidase.

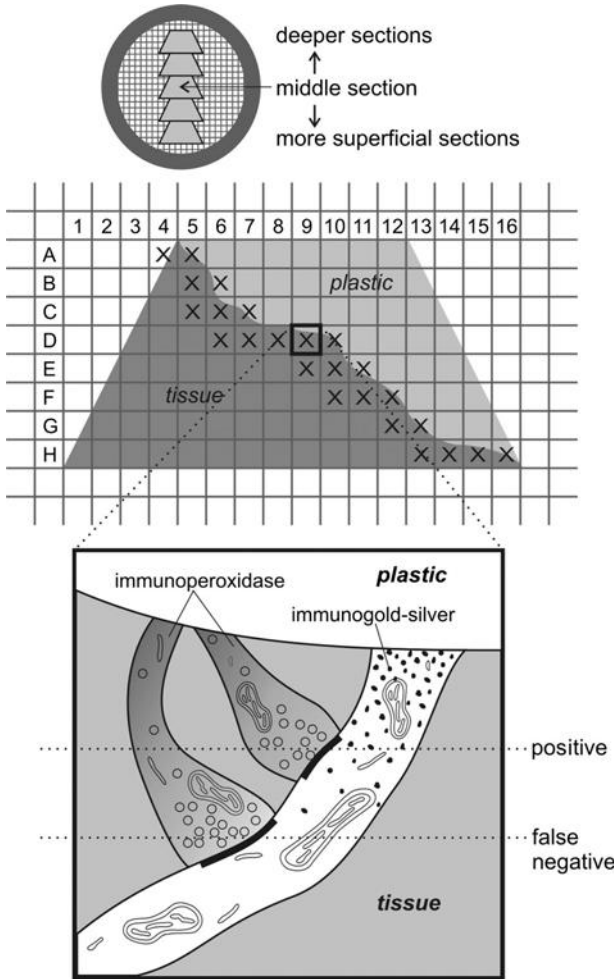
## G. Tissue Sampling

### 1. General Sampling Issues

In preembedding tissue labeled by immunoperoxidase or immunogold-silver, it is crucial to examine only the surface of sections, which is represented by the interface between the tissue and the plastic embedding medium. Because large amounts of penetration enhancers are not feasible for immunolabeling of tissue for TEM, antibodies do not have access to the full extent of the section thickness. Thus, maximal antibody penetration is limited to a few microns of the tissue surface. Moreover, even if one restricts sampling to the tissue surface, the actual protein levels will be underestimated with these techniques, due to the chemical fixation and low concentrations of penetration enhancers. Hence, immunocytochemistry for TEM involves a compromise that balances immunodetection with morphological integrity. Immunoreactivity that is excessive on the surface may not penetrate more than a few microns (Fig. 2.16). Conversely, morphological preservation may be excellent within the depths of the tissue section but compromised at the surface where antibody penetration is greatest. Moreover, the extent to which immunoreagents penetrate the tissue is not equal between methods, with preembedding immunogold-silver generally penetrating less well than immunoperoxidase (Fig. 2.16) (Chan *et al.*, 1990).

Knowing that immunoreactivity is confined to the outer surface of flat-embedded sections, the investigator must decide what approach to take regarding ultramicrotomy. Some investigators actually turn their thick sections on edge and cut ultrathin sections perpendicular to (i.e., at 90° from) the original plane of sectioning. This approach has the advantage of allowing the experimenter to measure precisely the distance from the tissue surface where immunolabeling becomes weak or nondetectable. However, in addition to being a considerable technical challenge, this method has the disadvantage of causing lost perspective regarding how the area being sectioned relates to typical LM views of the region of interest. It is also possible to cut the tissue surface at an oblique angle so that the zone containing optimal antibody penetration and tissue morphology is “stretched” over many sections. However, in our experience, this reduces the size of the useful zone within each ultrathin section and creates the need to collect many more sections in order to obtain a sufficient sample size. Hence, our approach has always been to embed the thick sections in plastic as flat as possible (by placing them under glass slides and heavy lead bricks) and to perform ultramicrotomy en face.

In order to accomplish the goals of analysis at the tissue surface, we further recommend collecting ultrathin sections on grids that contain at least three sections, each with part tissue and part embedding resin. Focusing the analysis on the middle section allows serial examination in sections both deeper and more superficial to the central one (Fig. 2.16). Using mesh grids allows the creation of a grid map for estimating the area analyzed and relocating



**Figure 2.16.** Schematic diagrams showing the sampling strategy used for preembedding immunoelectron microscopic studies (see text, section “General Sampling Issues”).

regions of interest during serial section analysis or in the event that better photomicrographs are required. Mesh grids are also generally tougher and stand up well to repeat handling. Of course, loss of desired regions under the metal mesh is inevitable with these grids. If this becomes a problem for a study, then slot grids are recommended so that all of the tissue is supported on a nonobscuring film like formvar. However, slot grids are generally more fragile and it can be difficult to relocate specific areas of interest if it becomes necessary after a viewing session.

At low magnification, it is advisable to take a photomicrograph or make a drawing of the ultrathin section and assign letter and number coordinates to each grid square. At  $3000\times$  magnification, our laboratory chooses

grid squares that (a) contain at least 25%, but no more than 75% tissue or (b) share an edge (not a corner) with a square that meets the first criterion (Fig. 2.16). These criteria ensure that the TEM sample is collected at a relatively uniform distance from the tissue surface. Recording which grid squares are to be analyzed and the approximate amount of tissue that each contains (25, 50, 75, or 100%) allows estimation of the area of tissue analyzed. For example, thin bar copper 400 mesh grids (Electron Microscopy Sciences) have squares that are 55  $\mu\text{m}$  on a side, or 3025  $\mu\text{m}^2$  area. This is then multiplied by the number of squares analyzed and the proportion of tissue that each square contained to derive an estimate of the total tissue area sampled.

Selected squares are then analyzed at higher magnification (10–30,000 $\times$ ). For each square, it is good to record the number of fields that contain specific peroxidase and/or specific gold–silver labeling. We define a “field” as the area delimited by the photographic brackets on the microscope. In this case, either fields that are photographed or simply analyzed for content without photo documentation can be recorded for the number of events per unit area (area of the bracketed region). In the case of receptor or transporter studies, this gives an estimate of the density of immunolabeling (i.e., number of labeled profiles per unit area). Profiles can be further analyzed for the position of gold–silver particles in relation to the plasma membrane or other structures. For synaptic connectivity, fields are analyzed for whether labeled processes (usually axons) contact unlabeled or labeled targets (usually soma, dendrites, or spines) or make no obvious contacts. A coordinate relocation system on the microscope can be used to examine serial sections to verify whether labeling of profiles is specific (e.g., number of gold–silver particles per profile or per unit area) and whether synaptic specializations are present at points of contact.

## 2. Criteria for Immunoperoxidase

In general, specific labeling using immunoperoxidase staining is easy to discern as a flocculent, dense precipitate within labeled structures. Occasionally, nonspecific or “background” labeling may also be present. Unfortunately, there are no established principles for estimating the amount of background staining in immunoperoxidase-labeled tissue. In fact, in some instances, nonspecific labeling may be somewhat difficult to determine, because it is typically related to the primary antibody. In other words, if the primary antibody is omitted, the background staining disappears. It is for this reason that simply omitting the primary antibody is not an acceptable test for specificity; rather, it is merely a control for the specificity of the secondary antibody. Specific immunoperoxidase staining should be confined to structures that have the potential to synthesize the antigen. For instance, labeling in cells that do *not* express the mRNA for the protein has a high likelihood of indicating nonspecific or background reactivity and must be examined with caution. Background labeling also tends to be more diffuse

and less dense than specific peroxidase product. Nevertheless, we have often noted that diffuse cellular immunoperoxidase labeling that is visualized by LM is not always evident by TEM. Conversely, TEM may detect immunoreactivity that is too weak to produce a visible signal in LM. In any event, the presence of suspected background labeling necessitates rigorous tests for specificity of the primary antibody.

### 3. Criteria for Immunogold–Silver

Immunogold–silver labeling is sometimes associated with a higher probability of background labeling than is immunoperoxidase, necessitating the establishment of consistent criteria for determining specific immunoreactivity. Such criteria will depend on the localization and density of the antigen as well as the level of background immunogold–silver labeling. Basically, experimenters should be confident that their criterion for specific immunogold–silver labeling would not be met by randomly distributed gold–silver particles throughout the tissue, and this in turn is affected by the size of the structure of interest. For example, the probability that three gold–silver particles would distribute randomly within a proximal dendrite is high, but the presence of three random particles within an axon terminal is a low probability event. Similarly, the probability that two gold–silver particles within an axon terminal would both be randomly distributed to the plasma membrane is a low probability event compared to the same particles localized to the cytoplasm. Hence, for our published studies of plasma membrane transporters, we have set our criteria for specific immunogold–silver labeling within axons as at least two particles on the plasmalemma or three in total (including those in the cytoplasm) (Miner *et al.*, 2000, 2003c). These criteria are somewhat more conservative than other laboratories (Garzón *et al.*, 1999; Pickel and Chan, 1999), and so we must acknowledge a higher likelihood of false-negative outcomes in our studies. On the other hand, we can more confidently assert the absence of false-positive results.

## IV. SUMMARY OF ADVANTAGES AND LIMITATIONS

### A. Advantages

Preembedding immunoperoxidase methods involving signal amplification have the advantage of superior sensitivity for the localization of sparse antigens. This is particularly true for TEM, as the greater resolving power of electron microscopy allows detection of low levels of peroxidase reaction product that may appear too diffuse for LM identification. The more discrete and nondiffusible marker associated with the preembedding immunogold–silver technique provides the advantage of indicating the precise subcellular localization of antigens, including neurotransmitter receptors and transporters. Discrete gold particles, with or without silver enhancement, are also

advantageous because they are more readily quantified than the precipitate formed by peroxidase reaction. When combined, the immunoperoxidase and immunogold methods provide a powerful means for localizing two or more antigens in relation to each other. With TEM detection, these approaches allow the identification of specific synaptic relationships formed between cellular elements that are labeled by tract-tracing and/or neurochemical phenotype. If two antigens are known to be localized to separate neuronal compartments (e.g., anterograde and retrograde tracing agents in dendrites and axons, respectively), then the combination of preembedding immunoperoxidase and immunogold methods can be used for triple-labeling studies, specifically the determination of whether afferents into a region of interest synapse onto populations of cells identified both by their neurotransmitter phenotype and major axonal target.

### **B. Limitations: Sources of False-Positive Errors**

For any TEM study involving tract-tracing and immunocytochemistry, consideration must be given to potential sources of false-positive and false-negative results. Common sources of false-positive errors are cross-reaction of the primary antibody with unknown proteins, cross-reaction of secondary antibodies with inappropriate species, nonspecific immunolabeling, or erroneous transport of tract-tracing agents. The evidence supporting specificity of immunoreagents for TEM is the same as for LM studies and should be demonstrated with appropriate controls prior to the experiment. Much has been written on this subject and on sources of nonspecific/background labeling. The reader is referred elsewhere for a consideration of these issues (Saper, 2003) (see also section “Antibody Controls”).

For experiments involving tract-tracing, the investigator should be familiar with the LM literature on afferents and efferents of each region of interest and hence the potential sources of false-positive results if tracer injections spread beyond the target. Such cases should be eliminated if tracer spread would involve an adjacent pathway that is not the one under study. Another common source of false-positive labeling in tract-tracing studies of the type described here is uptake of retrograde tracer by fibers of passage. The extent of this problem varies with different retrograde tracers, but the tracer with the least amount of uptake into passing fibers is PRV. If performing retrograde tract-tracing from a region in which uptake by fibers of passage is likely to produce false-positive results, it is recommended that PRV be used, and that potential contributors to false positives are systematically checked in control experiments. Finally, and as described above, use of BDA for anterograde tracing has the potential to produce false-positive results if it undergoes retrograde transport and subsequent anterograde trafficking into collateral axons. When using BDA, care should be taken to examine the most likely brain regions in which retrograde transport might occur. Evidence of retrograde transport indicates that collateral transport

is a possibility; such cases should be discarded, and PHA-L should then be used as the preferred anterograde tracer.

### C. Limitations: Sources of False-Negative Errors

False-negative results are a common concern in studies using TEM immunocytochemistry due to the limited penetration of immunochemicals in tissue prepared with minimal or no detergent. This drawback especially affects the detection of immunogold reagents, which typically penetrate less deeply into the tissue than immunoperoxidase compounds (Chan *et al.*, 1990). Although these limitations cannot be completely avoided, their impact can be minimized by utilizing the less sensitive immunogold–silver method to label antigens in high abundance, confining the analysis to the surface of the sections where both peroxidase and gold–silver markers are present, and analyzing serial sections for all profiles with sparse labeling. Anterograde and retrograde tract-tracing can also contribute to false-negative results, because all neurons in a population of interest are unlikely to be labeled in any given study. The use of multiple animals with slightly different injection sites can help to overcome this limitation, as can the use of large injections where possible. Finally, for retrograde tract-tracing with FG, an additional source of false-negative results discussed earlier (section “Retrograde Tract-Tracing”) is that the tracer may be confined to lysosomes and not spread diffusely within dendrites. Extensive sampling, examination of dendrites in serial section, and following dendrites cut longitudinally to see whether lysosomes are present can help to minimize this problem.

An additional methodological consideration for the methods presented here is the ability to detect the presence of multiple markers within one profile. Unfortunately, the limitations of preembedding immunocytochemistry are such that it may not be possible to detect the colocalization of markers in every structure in which these antigens are present, particularly when the neuronal structures are small. This limitation may result from imperfect antibody penetration, spatial interference when relatively large reagents compete for access to antigens within confined spaces, differential location of antigens (e.g., cytoplasmic versus vesicular), disproportional concentration of antigens, and unequal sensitivity of immunoperoxidase and immunogold–silver methods. In our experience, such impediments typically have a greater impact on the dual labeling of axon terminals as opposed to dendrites. Sometimes, the problem can be addressed by switching the order in which immunoperoxidase and immunogold labeling are performed (Katona *et al.*, 2001) or using a sequential method of incubation in primary antibodies rather than the parallel method recommended here. Adjusting the concentrations of the primary antibody may also help, for example, reducing the concentration of the antibody directed against the antigen in greatest abundance. However, this issue remains a limitation of preembedding methods and one for which postembedding methodologies (Charara



*et al.*, 1996; Smith *et al.*, 1996) may be superior for avoiding false-negative outcomes.

## V. PROSPECTS FOR THE FUTURE

It is hoped that microarray studies (see chapter by Ginsberg *et al.*) will eventually identify unique phenotypic markers for neuronal pathways and so render obsolete the need for tract-tracing. Having such protein markers would avoid the need for survival surgery in animals. Moreover, identifying pathways based on unique protein signature is likely to have greater sensitivity compared to tract-tracing, in that antibodies have the potential to detect all the neuronal structures that contain that protein, whereas tracer injections virtually never include all the cells and/or processes that contribute to a given projection. Of course, limited antibody penetration will still present difficulties for interpretation of TEM studies of protein localization or synapse identification. A second potential solution to the limitations of tract-tracing would be the introduction of genetically altered animals in which marker proteins (e.g., green fluorescent protein) are expressed in cells of interest (Zhao *et al.*, 2004) that are otherwise difficult to study by standard methods, for example GABA neurons that project from the VTA to the PFC. The ability to be certain that the marker protein is expressed in all such cells and their processes will allow a more complete analysis of their synaptic organization at both cell body and nerve terminal levels.

## APPENDIX

Here, we will present a full protocol for the immunoperoxidase and immunogold–silver procedures for TEM. This can be followed for single-, dual-, or triple-labeling experiments.

### A. Recipes for Standard Buffers

#### 1. 0.2 M Sodium Phosphate Buffer

1000 ml distilled water  
21.8 g sodium phosphate dibasic  
6.4 g sodium phosphate monobasic  
pH to 7.3 with phosphoric acid, not HCl (see section “Osmication”)  
Dilute 1:1 with water to make 0.1 M sodium phosphate buffer (PB)

#### 2. 0.01 M Phosphate-Buffered Saline (PBS)

1000 ml distilled water  
50 ml 0.2 M PB  
9 g sodium chloride

### 3. 0.1 M Tris-Buffered Saline (TBS)

1000 ml distilled water  
12.1 g trizma base  
9 g sodium chloride  
pH to 7.6 with HCl

## B. Fixation

### 1. Rat Preparation for Immunogold Procedure

Animals are first anesthetized with 60 mg/kg pentobarbital i.p. and then given 1 g/kg i.p. of diethylthiocarbamic acid (Sigma, St. Louis, MO) for 15 min prior to aldehyde perfusion. During this treatment, animals should be carefully monitored for seizures, which can be induced by chelation of zinc. In the event that seizure activity is detected, animals should be given supplemental doses of anesthetic.

### 2. Perfusion and Fixatives

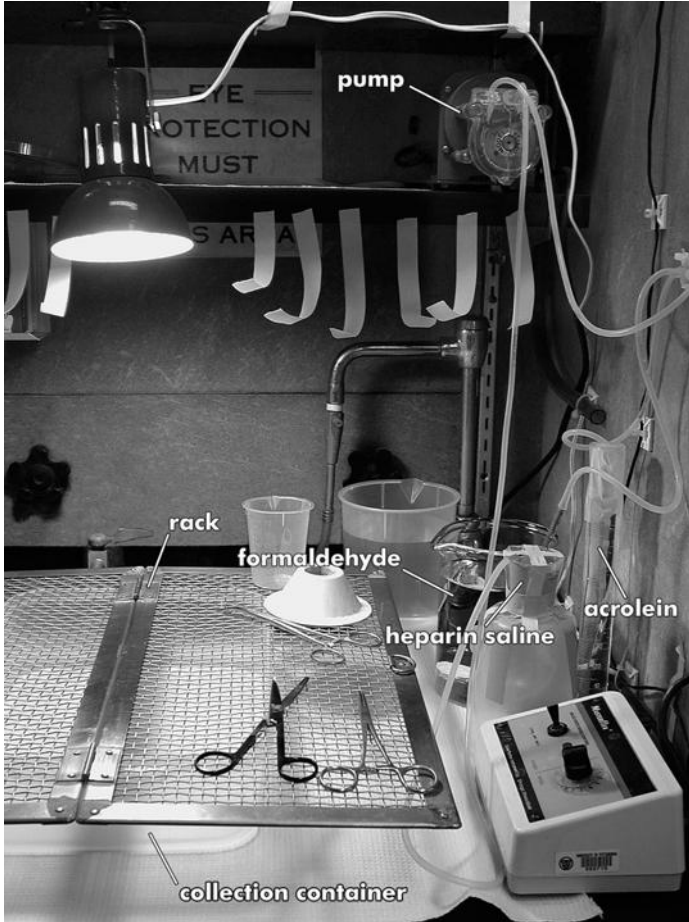
Correctly prepared fixatives for perfusion should be filtered and then checked to ensure that they are clear and colorless.

#### a. 3.8% Acrolein, 2% Formaldehyde in 0.1 M PB

Rats are perfused with 10 ml heparin saline (1000 U/ml; Elkins-Sinn, NJ), followed by 50 ml of 3.8% acrolein and 2% formaldehyde, followed by 200–400 ml of 2% formaldehyde in 0.1 M PB. Coronal blocks of brain are postfixed in 2% formaldehyde for 30–60 min.

In a well-ventilated hood, prepare 2% formaldehyde in 0.1 M phosphate buffer as follows. Heat 500 ml of ultrapure water in a 1-l glass beaker to 60–65°C. Do not exceed 65°C. Turn off the heat and add 20 g of EM grade granular paraformaldehyde (Electron Microscopy Sciences; Fort Washington, PA), stirring constantly. Stir for several minutes and then add small volumes of 1 N NaOH, stirring for several minutes after each addition until the solution is mostly clear. A few granules of paraformaldehyde might still be present. Filter through a Buchner funnel and an aspiration flask using #3 filter paper. Also filter 500 ml of 0.2 M PB. Transfer the solution to a beaker.

For one rat, measure 48.1 ml of the 2% solution of freshly depolymerized paraformaldehyde (i.e., formaldehyde) into a 100 ml graduated cylinder. We recommend the use of acrolein from Electron Microscopy Sciences because it is supplied in 2 ml single-use glass ampoules and so does not require storage of opened containers of acrolein. Wearing gloves and eye goggles use a 5-cm<sup>3</sup> syringe and an 18-G needle (the large gauge is needed because of the high vapor pressure) to remove 1.9 ml of acrolein from the glass

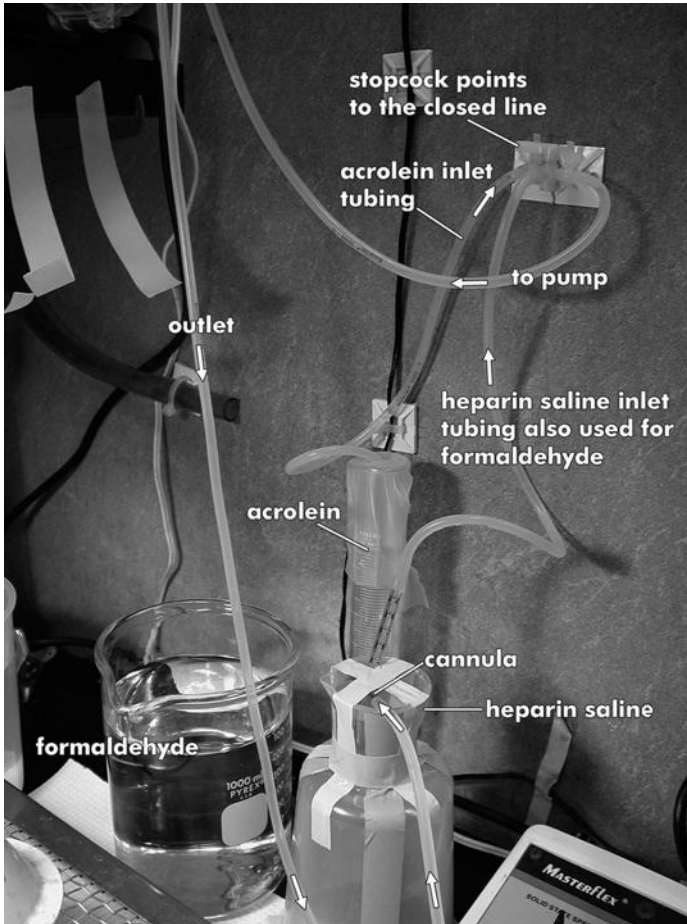


**Figure 2.17.** Equipment setup for intracardial perfusion with acrolein by using a peristaltic pump. The rack above a collection container allows recovery of perfusate for safe disposal, and the entire assembly is placed within a laminar flow hood to contain acrolein vapors.

ampoule. Add acrolein to the graduated cylinder; seal tightly with parafilm and invert several times to mix. For two rats, use two ampoules of acrolein and add 3.8 ml of acrolein to 96.2 ml of 2% formaldehyde.

The perfusion system that is needed for this fixative consists of a peristaltic pump and tubes that are attached to a three-way stopcock with two inlets and one outlet, all assembled in a well-ventilated laminar flow hood (Figs. 2.17 and 2.18). This system allows the delivery of two to three different solutions without the introduction of air bubbles that might block brain capillaries. Although not shown in the figure, we recommend using a metal clamp stand to ensure that the graduated cylinder containing acrolein does not inadvertently tip over.

The rat is placed on a perfusion rack that is set over a plastic container in order to collect the blood with acrolein perfusate. Full descriptions of



**Figure 2.18.** Tubing arrangement for acrolein perfusion. The use of a three-way stopcock allows for two inlet lines and one outlet. The first inlet tube is primed for acrolein, and the stopcock is then switched to shut off the acrolein and allow flow through the second inlet tube. This is first rinsed with water to remove residual acrolein and then primed with heparin saline. The outlet tube is run into the heparin saline, which circulates while the animal's abdomen and thorax are exposed. The cannula from the outlet tube is then inserted through the base of the left ventricle into the aorta and clamped in place. The stopcock is then switched to allow acrolein to perfuse the animal. This shuts off the flow of heparin saline, making it safe to move the inlet tube from the heparin saline into the formaldehyde. Once the correct volume of acrolein is perfused, the stopcock is switched one final time to complete the perfusion with formaldehyde.

the surgical procedure can be found elsewhere (Friedrich and Mugnaini, 1981; Leranath and Pickel, 1989). Briefly, once the anesthetized rat's pain reflexes have ceased completely, tape down the arms and tail. Turn the peristaltic pump to run at a flow rate of approximately 50 ml/min. Cut a wide opening in the lower abdomen; find the xiphoid process and clamp with a regular hemostat. Holding the hemostat cut up either side of the

rib cage, cut open the diaphragm and remove any membrane surrounding the heart. Slit open the right atrium and then the base of the left ventricle. Push the outflow tubing with the cannula through the left ventricle and up into the aorta; clamp the cannula into the aorta with a vascular hemostat. The cannula can also be clamped onto the left ventricle if preferred, but in either case the hemostat should be propped to keep it from twisting. Immediately switch the stopcock to introduce the acrolein with 2% formaldehyde and turn up the perfusion speed to 90 ml/min; pump through 50 ml of acrolein. In the meantime, transfer the heparin inflow tubing into the plain 2% formaldehyde. After acrolein, switch the stopcock to pump through 200 ml of formaldehyde and turn down the pump speed to 80 ml/min.

When handling acrolein, always wear gloves and eye protection. Vials should only be opened and the perfusion should only be performed in a well-ventilated hood with the shield lowered. Acrolein remaining in the ampoules and any fluids containing acrolein after the perfusion is completed should be collected into a labeled glass waste container that is stored in a flammable liquids cabinet until it can be properly disposed. Use a funnel to empty the blood/perfusate into the glass storage bottle and rinse several times with water until the collection container is safe to remove from the hood for final cleaning. Any items that contact acrolein (vials, ampoules, syringes, etc.) should be kept in the hood at least overnight until the solution has evaporated (even a small amount of acrolein put in the trash will soon become evident to anyone in the room). If a spill occurs outside the hood, evacuate the room immediately and call chemical safety. If possible, turn on the hood, as this may help to clear the acrolein vapors and contain them to the affected room. Use a safety shower to wash any skin or clothing that contacts acrolein.

#### **b. 0.05–1% Glutaraldehyde, 4% Formaldehyde in 0.1 M PB**

Rats are perfused first with heparin saline as above, followed by 500 ml of the para/glut fixative. Coronal blocks are postfixed in 4% formaldehyde for 30–60 min.

In a well-ventilated hood, prepare 4% formaldehyde in 0.1 M phosphate buffer as follows and as described in detail above. Heat 500 ml of ultra-pure water. Add 40 g of EM grade granular paraformaldehyde, followed by small volumes of 1 N NaOH until clear. Filter solution, followed by 500 ml of 0.2 M PB. Transfer the solution to a beaker. For the standard 0.2% glutaraldehyde, add 8 ml of 25% EM grade glutaraldehyde to a liter of the 4% freshly depolymerized paraformaldehyde.

### **3. Vibratome Sectioning**

After perfusion, remove the brain from the skull and cut it into thick blocks that contain the brain regions of interest. Postfix for the times

described above, and then transfer to 0.1 M PB and section on a Vibratome. For the best sectioning, use well-fixed brains, fill the Vibratome with 0.1 M PB that has been cooled to 4°C, use fresh, sharp blades, and set the slicer to low speed and wide amplitude. Section at 40–60 µm and collect sections in serial order into cell wells containing 0.1 M PB.

#### 4. Sodium Borohydride and Hydrogen Peroxide Treatments

Rinse sections in 0.1 M PB and divide into multiple conditions as desired. Incubate sections for 30 min in 1% sodium borohydride in PB. As sections will float to the top, maintain hydration by mixing them back down occasionally. Rinse sections extensively in PB until all bubbles are gone. If endogenous peroxidase activity has the potential to confound interpretation in the study, treat sections for 15 min with 3% H<sub>2</sub>O<sub>2</sub> in 0.1 M TBS and then rinse extensively in this buffer.

#### 5. Cryoprotection and Tissue Storage

For long-term storage in a solution that is compatible with later EM analysis, place sections into the following cryoprotectant (generously provided by Darlene Melchitzky) and freeze at -20°C. Other potential cryoprotectant solutions may also serve this purpose (Rosene *et al.*, 1986).

Storage cryoprotectant

- 300 ml ethylene glycol
- 300 ml glycerol
- 100 ml 0.2 M PB
- 300 ml ultrapure water

### C. Immunolabeling

Immunolabeling procedures are performed on free-floating sections at room temperature with constant shaking unless otherwise specified.

#### 1. Primary Antibody Steps and Penetration Enhancement

- a. **Optional, Freeze–Thaw Procedure** (protocol generously provided by Dr. Yoland Smith)

Place sections in cryoprotectant for 20 min.  
Freeze thaw cryoprotectant

200 ml 0.2 M PB  
520 ml distilled water  
80 ml glycerol  
200 g sucrose  
qs final volume to 1000 ml  
Solution can be stored in  $-20^{\circ}\text{C}$  freezer.

Place sections into  $-80^{\circ}\text{C}$  freezer for 20 min.

Thaw sections at room temperature for 10 min each in the following solutions: cryoprotectant at 100, 70, 50, and 30% diluted in PBS.

Rinse sections in PBS ( $3 \times 5$  min).

Rinse sections in TBS ( $3 \times 5$  min).

### **b. Blocking Solution**

87 ml TBS  
10 ml 0.4% Triton X-100 (Sigma) (optional)  
Final concentration is 0.04% Triton.  
3 ml normal serum  
1 g BSA

Place sections in blocking solution for 30 min.

Incubate sections in primary antibody made up in blocking solution overnight at room temperature or over two nights at  $4^{\circ}\text{C}$ .

Rinse sections in 0.1 M TBS (1 min then  $3 \times 10$  min).

If single labeling for immunogold–silver is being performed, proceed to section “Immunogold Labeling.” Otherwise, follow the next steps.

## **2. Immunoperoxidase Labeling**

Mix biotinylated secondary antibody in blocking solution. Typical final concentrations are 1:100 to 1:400.

Incubate sections in secondary antibody for 30 min.

Mix ABC complex solution using the Vectastain Elite kit (Vector Laboratories) by adding two drops each of A and B solutions to 10 ml of 0.1 M TBS. Allow to stand at least 30 min before use. Take care not to contaminate the dropper bottles and do not overmix or vortex the solution.

Rinse sections in 0.1 M TBS (1 min then  $3 \times 5$  min).

Incubate sections in ABC solution for 30–120 min.

Rinse sections in 0.1 M TBS (1 min then  $3 \times 5$  min).

Prepare the DAB (Sigma) immediately before use. To 100 ml of 0.1 M TBS, add 22 mg of DAB and  $10 \mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$ . Filter the solution.

Incubate sections in DAB for 3–6 min, depending on the desired strength of the reaction, keeping in mind that copious labeling in the light microscope may appear overblown by TEM.

Stop the reaction by rinsing sections in 0.1 M TBS (1 min then  $3 \times 5$  min).

For tissue to be plastic embedded after immunoperoxidase labeling, transfer sections to 0.1 M PB ( $2 \times 5$  min) and proceed to section "Tissue Preparation for Electron Microscopy." Otherwise, follow the next steps.

### 3. Immunogold Labeling

Rinse sections in 0.01 M PBS (1 min then  $3 \times 5$  min).

Place sections in washing buffer for 30 min.

Washing buffer

93.5 ml 0.01 M PBS

0.8 g BSA

0.5 ml fish gelatin (comes with the gold secondary antibody)

6 ml normal serum

Incubate sections in gold-conjugated secondary antibody diluted 1:50 in washing buffer for 2–4 h or overnight.

Rinse sections in washing buffer (1 min then  $3 \times 5$  min).

Rinse sections in 0.01 M PBS (1 min then  $3 \times 5$  min).

In a ventilated hood, fix sections in 2% glutaraldehyde in PBS for 10 min.

Remove silver enhancement solutions from the refrigerator (see below).

Rinse sections in 0.01 M PBS (1 min, then  $3 \times 5$  min) until odor of glutaraldehyde is gone.

0.2 M sodium citrate buffer

100 ml ultrapure water

5.88 g sodium citrate dihydrate

pH to 7.4 using citric acid

0.2 M citric acid

100 ml ultrapure water

4.2 g citric acid monohydrate

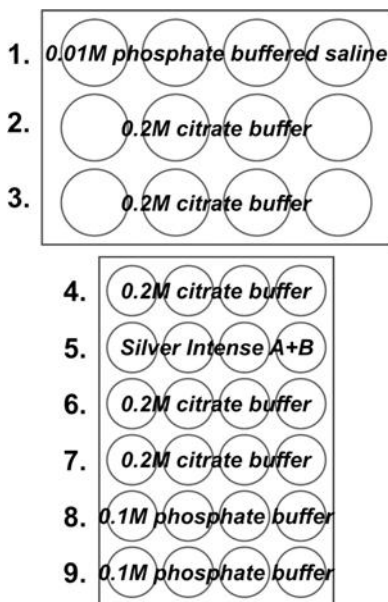
Keep refrigerated.

### 4. Silver Enhancement

Fill a 12-well cultured cell plate and a 24-well plate as shown in Figure 2.19. For the 0.1 M PB in the last two rows of wells, make sure to use solution that is not contaminated by chloride ions, as these are the final steps before osmication. Divide up the tissue in batches in the first row of 0.01 M PBS depending on the number of silver enhancement times to be tested. Do not proceed until all the wells (except silver) are filled with solution and the silver enhancement solutions are at room temperature. Do not allow sections to sit for long periods in the citrate buffer, as this has only weak buffering capacity that is not optimal for morphological preservation.

Once all is ready, fill the second row of the 24-well culture plate with equal drops of IntenSE M kit A and B (Amersham, Arlington Heights, IL)





**Figure 2.19.** Schematic illustration of cell culture wells showing the order of reagents used for silver intensification at different time points.

solutions. The exact volume will depend on the number and size of the sections, but usually 10 drops each of A and B are sufficient for 4–8 sections.

Using a wooden applicator stick with a pointed end (snap in half from the ends to get a clean break), transfer sections to 0.2 M sodium citrate buffer in the second row of the 12-well cell plate. Rinse sections for 1 min. Using a new wooden applicator stick (to minimize contamination by phosphate), transfer sections to the third row of the 12-well cell plate and rinse for 1 min. Now transfer sections to the citrate buffer in the first row of the 24-well cell plate. Rinse for 1 min. Then transfer sections to the silver solution (second row of the 24-well cell plate) and start a timer.

Gently swirl the plates and time the silver enhancement reaction carefully, as differences of 30 s are significant. If needed, use a dissecting microscope to watch the progress of silver intensification. Stop the silver enhancement reaction by rinsing in buffer. Do not return sections to the silver once they are removed. Transfer sections into the third through sixth rows of the 24-well cell plate in succession (1 min each for citrate buffer and longer for PB). Sections can remain in the final row of 0.1 M PB until dishes for osmication are ready.

In practice, we have found it useful to perform the steps above for a pilot determination of optimal silver enhancement times on a small number of sections (see also section “Choice of Markers”). We usually run test sections at wide intervals (e.g., 4, 8, and 12 min), mount these on slides, and examine them by LM. The most optimal silver enhancement time is that

which produces clearly labeled neuronal elements, typically with a golden-brown color, with minimal background (e.g., few silver-enhanced gold particles in the white matter). Pale gold labeling is probably underdeveloped; black labeling is considered overdeveloped and likely to be associated with greater background particles (see also Chan *et al.*, 1990). For the actual silver enhancement of the experimental sections, we recommend dividing the sections into half and running them at two different times, 1–2 min apart. Subsequent TEM analysis may reveal that one of these tissue sets has better labeling characteristics with regard to specific and nonspecific labeling.

#### D. Tissue Preparation for Electron Microscopy

Rinse sections in 0.1 M PB ( $2 \times 5$  min) in Coors dishes. Place dishes in a well-ventilated hood. Prepare 2% OsO<sub>4</sub> in 0.1 M PB by mixing equal volumes of 4% OsO<sub>4</sub> and 0.2 M PB. Make sure sections are lying flat in the Coors wells then slowly draw off the phosphate buffer without disturbing the sections. Gently add OsO<sub>4</sub> taking care not to twist or fold the sections. Incubate in OsO<sub>4</sub> for 1 h in the hood.

The remaining steps for tissue preparation are standard in electron microscopy (Friedrich and Mugnaini, 1981) and will not be presented in detail here. Briefly, after rinsing several times with PB, sections are dehydrated at 10-min intervals through a standard series of increasing strength ethanol solutions (30%, 50%, 70%, 95%, 100%, 100%), then twice in 100% propylene oxide or acetone, followed by embedding resin equally mixed with propylene oxide or acetone. The sections are left for several hours or overnight and then infiltrated with pure resin for 2–4 h. Sections are then embedded between sheets of commercial plastic. Once polymerized at 60°C for at least 18–24 h, the resin-embedded sections are trimmed and cut on an ultramicrotome, and ultrathin sections are collected onto mesh or coated slot grids.

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