Morphological details of primate axons and dendrites revealed by extracellular injection of biocytin: an economic and reliable alternative to PHA-L

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INTRODUCTION

Our understanding of neuronal function can be enhanced greatly by knowledge of fine details of their axonal and dendritic processes. The problem is that many of the methods developed to reveal such details are limited in their usefulness. Several examples will suffice to make the point. The Golgi methods, although very useful for revealing dendritic morphology, are limited in that one cannot select the population of cells to be labeled in advance. Another problem with this method is that adult axons are not well labeled. In order to label only axon arbors, several investigators have mechanically severed axons proximal to their terminal arbors and filled these cut ends (by diffusion) with horseradish peroxidase (HRP)⁹,¹⁰,¹⁶,¹⁹,²²,²⁸. This method, while providing an excellent view of the terminal arbors, suffers from one major shortcoming: it cannot account precisely for the axon's origin. The problem of determining an axon's origin can partially be solved by impaling the axon with a recording pipette, characterizing its physiology and then filling it iontophoretically with HRP²,²⁸. However, this technique is expensive, requires great skill, is time consuming and results in a very small sample. In addition, the origin of cells can only be indirectly inferred from the cell's physiology. The latter method has also been used to directly reveal dendritic morphology by injection of HRP or other labels into cells both in vitro and in vivo. However, since axons of intracellularly filled cells are rarely labeled well enough to be traced to their terminal points, one can never directly determine the destination of the cell's output.

A recently introduced technique that uses the lectin Phaseolus vulgaris leucoagglutinin (PHA-L) promised to solve the more difficult problems associated with revealing axonal arbor detail²⁹. This lectin can be injected iontophoretically, is taken up extracellularly by cells and is then transported anterogradely to fill distant axon arbors revealing their configuration and finest detail. The lectin is visualized using the relatively straightforward avidin/biotin method of Hsu et al.¹⁴. Unfortunately, it has been learned by trial and error by a number of investigators that PHA-L only reliably labels axon arbors in a few species. To date, only a handful of investigators have successfully used PHA-L in mammals other than rodents, (e.g. cats⁵,⁵⁰). Few laboratories (to our knowledge) have been able to successfully use this lectin in primates with modifications to the original protocol of Gerfen and Sawchenko¹¹,¹⁷,²⁴,²⁵,²⁷.

Biocytin, originally used as an intracellular marker¹³, is a conjugate of biotin and the amino acid, lysine. Recently, King et al.⁹, following injection parameters used...
by Gerfen and Sawchenko\textsuperscript{11} for PHA-L, have lauded biocytin as an alternative to Phaseolus. However, King et al.\textsuperscript{15}, like Gerfen and Sawchenko\textsuperscript{11}, used the rat to test the efficacy of biocytin as an anterograd e tracer. In this report, we have tested the tract-tracing and axonal and dendritic filling capacity of biocytin in several primate species. The results show that biocytin can be used in these species. As with Phaseolus, some procedural modifications are recommended. Our results show that biocytin is an excellent anterograd e tracer in primates, which fills axon terminals completely. It also serves, contrary to reports in rats\textsuperscript{15}, as an excellent retrograde marker that is far superior to horseradish peroxidase (HRP) in its ability to delineate the morphology of dendrites. We have also found that the biocytin method is compatible with cyt crome oxidase (CO) histochemistry and with immunocytochemical procedures.

** MATERIALS AND METHODS **

** Subjects **

In this study we used 3 Old World macaque (cynomolgus) monkeys (Macaca fuscata), 3 New World squirrel monkeys (Saimiri sciureus), 4 Old World prosimian bushbabies (Galago crassicaudatus) and 4 tree shrews (Tupaia belangeri). Tree shrews have been variously classified as insectivores, primates, or in their own order\textsuperscript{12}.

** Surgery and recovery **

Surgical procedures were carried out on deeply anesthetized animals (ketamine supplemented with xylazine according to species recommended dosages) under sterile conditions according to procedures described previously\textsuperscript{16}. All injections (see below) were made using stereotaxic coordinates established for each location in previous studies. Following surgery, animals were given a long-acting antibiotic and were carefully monitored until they were awake and capable of eating and drinking on their own.

** Injections **

In the 3 macaque monkeys, 1 pressure injection (2 hemispheres) or approximately 20 iontophoretic injections (2 hemispheres) were made into striate cortex, and 2 pressure injections (1 hemisphere) were made into the somatosensory cortex. Survival times varied from 24 h to 2 weeks. In the 3 squirrel monkeys, approximately 20 iontophoretic injections were made into the striate cortex in 3 hemispheres. Survival time was 1 day in each case. In the 4 bushbabies, 2–3 iontophoretic injections were made in 1 superior colliculus (SC), approximately 20 iontophoretic injections were made in the striate cortex in 1 hemisphere, and 1 or 2 iontophoretic injections were made into the lateral geniculate nucleus (LGN) in 3 hemispheres. Survival time was 2–5 days. In the 4 tree shrews, 2–3 iontophoretic injections were made in the superior colliculus in 3 hemispheres and 3–4 iontophoretic injections were made in the striate cortex in 4 hemispheres. Survival times varied from 9 hours to 3 days. Two of the 4 brains were also reacted to reveal choline acetyltransferase (ChAT).

For these experiments, the biocytin (Sigma) was dissolved in 50 mM Tris buffer (pH 8.2), 50 mM phosphate buffer (pH 7.6), or 10 mM phosphate buffered saline (PBS) (pH 7.4) to a final concentration of 2.5–5%. All vehicles produced comparable results, but the higher concentration of biocytin generally produced better filling of axons and dendrites. We generally used a fresh solution, although a 1-day-old solution of biocytin did successfully label neurons on the few occasions that we used it. The biocytin solution sometimes became cloudy if stored for any length of time; if the biocytin came out of solution it was re-dissolved by placing the container of biocytin solution (in our case a Beem capsule) in warm tap water for a few seconds.

Iontophoretic injections of biocytin were made through glass pipettes with inner tip diameters of 5–50 μm, without having to significantly alter voltage to maintain a 0.5–7 μA injection current. Both square-wave pulse duty cycle pulses (1–3 s duration) and continuous current were used to expel biocytin. We used a constant-current power supply for iontophoretic injections, so any lesion maker or pulse stimulator with a DC option could be used. We have used stimulators that deliver AC current, but results were inconsistent. Injection durations ranged from as little as 1 min to trace intrinsic cortical circuitry to as long as 15–20 min to trace longer pathways (e.g. the LGN to striate cortex in a 2–5 kg primate).

Pressure injections of 0.1–0.3 μl of biocytin were made with a Hamilton syringe. The solution was expelled slowly over a 5–10 min period in the same manner that was used to make bulk injections of horseradish peroxidase\textsuperscript{18}.

To determine if biocytin was picked up and transported by fibers of passage we also injected biocytin into the optic tract and the optic radiations, using both iontophoretic and bulk injection methods. No evidence of transport was found in any of these cases, suggesting that biocytin is not taken up by cut axons. Hence, while the exact mechanisms of uptake are not known, it is possible that biocytin may be incorporated via an active mechanism, which might be regulated by membrane "receptors" or pumps located on the soma or dendrites. Since biocytin is a conjugate of biotin and lysine, it may be incorporated into cells by the amino acid uptake system\textsuperscript{19}.

** Survival **

Animals survived 9–24 h for tracing short connections and 2–14 days for tracing longer connections. Even the case with the shortest survival time revealed some labeled axons and cells that were several centimeters from the injection site suggesting that biocytin is either transported (via fast axonal mechanisms) or diffuses very quickly through the neuron.

At present, it is unclear whether the biocytin is degraded. It is clear from our material that there were no significant differences in the quality of stained neuronal processes (at either the injection site or the target) in animals that survived for 9 h versus those that survived 2 weeks.

** Fixation **

Animals were euthanized by an overdose of a barbiturate. Subsequently, a lactated Ringer's solution was perfused transcardially, followed immediately by a larger volume of 2-4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer (pH 7.4). Finally, the same fixative supplemented with 10% sucrose was perfused. Brains were removed and stored overnight in cold 30% sucrose in phosphate buffer.

No differences in biocytin labeling were evident between cases in which 2% versus 4% formaldehyde was used. Lower concentrations of formaldehyde were used to accommodate the flattening of cortical hemispheres for some of the cases. However, in 2 additional tree shrews fixed with a mixed aldehyde solution containing phosphate-buffered 4% formaldehyde and 1% glutaraldehyde, we could not find any biocytin-labeled neuronal elements and only a very small, weakly differentiated injection site. King et al.\textsuperscript{15} have successfully visualized biocytin-labeled neuronal processes in formaldehyde/glutaraldehyde-fixed rat hippocampus using incubation times that ranged from 2 to 72 h.

** Histology **

Frozen sections 40–60 μm thick were cut in the coronal, parasagittal or tangential plane on a sliding microtome and collected in PBS. Biocytin-labeled processes were visualized using a Vectastain ABC kit (Vector Labs). Freely floating sections were incubated in the ABC solution at room temperature under constant agitation.
In most cases we immediately reacted the sections containing injection sites using a Standard ABC (Vector Labs) solution for 20–30 min. It is important to emphasize that a Standard Vector kit be used (specifically, cat. no. PK-6000), not a kit designed to bind to a linking antibody (it is not clear why other kits do not work. Vector has had no helpful input on this subject). Sections containing retrogradely labeled cells and anterogradely labeled axons were incubated (at room temperature, with agitation) for a maximum of 0.5–1 hour using an Elite (cat. no PK-6100) ABC Kit. According to Vector, the Elite ABC solution is more sensitive than the Standard solution (less sensitive, Vector claims), and is best suited for localizing antigens present in very low amounts. Longer incubation times using the Standard kit do not produce the same quality and intensity of label as the Elite kit. It is interesting, however, that lower concentrations of the Elite solutions (for example, 2–3 drops of sol. A and B/10 ml PBS) were just as effective in staining processes as dilutions recommended by Vector (2 drops sol. A and B/5 ml PBS). Recent experiments have indicated that the use of streptavidin-HRP (Amersham) at a concentration of 1:100 in calcium magnesium-free Hanks (Sigma) to replace the ABC step may produce better results with lower background and more intense labeling.

Following appropriate incubation times, sections were rinsed thoroughly in PBS and the peroxidase component of the avidin-biotin complex was visualized using diaminobenzidine (DAB). For the latter, we used either of two modified versions of Adams' method1. The first method, employed for all of the primate material described in this paper, is as follows (see also Lachica and Casagrande2): 50 mg of DAB was dissolved in 100 ml of PBS, to which 26.6 ml of 30% H2O2 is added. If sections were not adequately rinsed prior to this step, the unbound AB-complex adhered to the sections, coating them with a grey/black residue. This debris could be removed by sonicating sections briefly (30–60 s). The oxidation/reduction reaction proceeded for approximately 10 min before the addition of 500 µl of 1% nickel ammonium sulfate and 500 µl of 1% cobalt chloride per 100 ml of DAB solution. The addition of these metals changed the color of the reaction product from a red dish brown to a deep black, which was easier to visualize and photograph. When the color of the reaction product was satisfactory (approximately 3–5 min), sections were thoroughly rinsed in PBS. In order to reduce background label, sections were sometimes run first through DAB + H2O2, then transferred to a PBS/heavy metal solution (without the DAB) for intensification.

In the second procedure (which seemed to result in sections which are easier to mount) sections are rinsed 3 times in Tris buffer (pH 7.4) for 5 min each after the last PBS rinse. They are first incubated in 1% cobalt chloride and 1% nickel ammonium sulfate in Tris buffer for 15 min, rinsed 3 times, then transferred into DAB (0.05–0.02%) in Tris buffer where they remain for 15 min. Sections are next transferred to a fresh DAB solution containing 0.01–0.05% H2O2 and removed when the desired reaction is obtained, usually after 5–20 min. Sections are rinsed thoroughly with Tris buffer. This second procedure, used in conjunction with streptavidin-HRP (instead of ABC), has worked well for visualizing axonal axons in adult and developing ferrets.

If the intensity of the background label was unreasonably high following either of these procedures, it was reduced by treating free floating sections in a 0.1% buffered glutaraldehyde solution for 2–5 min. It is noteworthy that a typical method commonly used to reduce nonspecific staining for immunocytochemical reactions is to pretreat sections in a low concentration of peroxide. However, our experience shows that peroxide pretreatment is not compatible with biocyn histochemistry.

Following any of these procedures, all sections were mounted on gelatinized slides, air dried, defatted and coverslipped.

**Multiple labels: localizing biocyn and cytochrome oxidase on the same sections**

The cytochrome oxidase (CO) stain has proven to be a very important tool in marking physiologically and anatomically distinct regions of the brain3. For this study we attempted ‘double-label’ sections for biocyn and CO using 2 methods. In the first, which we prefer, sections were incubated in the ABC kit for 30–45 min, rinsed in PBS, then incubated in a CO solution (20 mg catalase, 30 mg cytochrome c, 50 µg DAB/100 ml PBS). Incubating sections at 45–50 °C decreased the time it took to adequately stain sections for CO, generally 45–60 min. Once stained, sections were rinsed with PBS and the HRP component of the ABC complex was visualized by placing the freely floating sections in a DAB + H2O2 solution (vide supra). The reaction progressed for 10–15 min, or until biocyn-labeled cells were easily differentiated against the CO stain. The reaction was terminated with several rinses of cold PBS. Biocyn-labeled cells appeared as a deep brown (almost black), against the red CO stained background.

In a second method, sections were incubated in ABC, reacted for DAB, then stained for CO. Following these procedures, the brownish-red biocyn-labeled cells were a little more difficult to visualize against the CO stain. When we intensified the reaction product with cobalt, the biocyn-labeled cells acquired a rich black color, but the background (i.e. the CO stain) also became darker.

**Multiple labels: combining biocyn and immunocytochemistry on the same section**

In 2 cases we combined procedures in order to localize both biocyn and ChAT labeled cells and processes in the same sections. That we elected to stain sections for ChAT is not important. We merely wanted to determine whether we could co-localize biocyn labeled and immuno-stained cells on the same section. It seems likely that we could have localized GABA or other antigens that can be visualized using standard immunohistochemical techniques.

For this procedure, sections were placed in PBS containing 1% normal rabbit serum (NRS) and the primary antibody (anti-ChAT, Boehringer Mannheim), diluted to a 1:50 concentration. The following day, sections were transferred to a PBS solution containing 1% NRS and the linking antiserum (biotinylated rabbit anti-rat IgG, Jackson Immuno) diluted 1:250. Freely floating sections were agitated in this solution for 30–45 min at room temperature, rinsed repeatedly in PBS, then incubated in a Standard Vector ABC (Vector Labs) solution for roughly 60 min. ChAT-positive cells and cells filled with biocyn were visualized using the peroxidase component of the ABC complex with DAB + H2O2 (vide supra).

**RESULTS**

**Biocyn as an anterograde label for axons**

Iontophoretic and bulk injections of biocyn using the parameters described above, were made in the SC, LGN, and striate cortex (area 17 or V1) to trace anterogradely labeled processes to the LGN, within striate cortex, and to area 18, respectively. In each case, the patterns of connectivity conformed with known projection patterns that have been described in the past using other tracing methods.

Anterogradely transported biocyn filled axons completely and uniformly, revealing the configuration of individual axon arbors in exquisite detail. These images are not comparable to any produced by tritiated amino acids, or by wheatgerm agglutinin conjugated to HRP (WGA-HRP), which reveal terminal arbors only as dense granule patches. With biocyn, the morphology of the finest individual telodendria was revealed and individual boutons were discriminated easily (Figs. 1 and 2). In most cases, a discrete bundle of axon terminals was la-
Fig. 1. A: shows a biocytin injection site restricted to the superficial gray layer of the tree shrew superior colliculus. The inset shows the injection site a higher magnification to illustrate that the central core of the injection site is well demarcated. A cell (arrow) adjacent to the injection site, whose axon or dendrites presumably lies within the injection site, is completely labeled. B: shows labeled collicular axon arbors in the ipsilateral lateral geniculate nucleus (LGN). As shown originally by Fitzpatrick et al., these axon arbors are restricted to the LGN interlaminar spaces, in this figure between layers 6 and 5, with a short branch in between layers 5 and 4. C: shows at higher magnification an individual branch from the colliculogeniculate arbor illustrated in panel B. Note that the finest branches are well filled and the boutons can be differentiated easily. SGS, upper division of stratum griseum superficiale; SGS.L, lower division of stratum griseum superficiale; SO, stratum opticum; OT, optic tract; numbers designate LGN layers. Dotted line in A marks the boundary between SGS and SO. Scale bar for A = 100 μm; for B = 30 μm; for C = 10 μm.

Fig. 2. A: shows an axon arbor in area 18 of macaque monkey filled via anterograde transport of biocytin following an injection in area 17. The portion of the axon within the box is shown at higher magnification in panel B. The arrow in panel A points to a small boutonal process which is also shown in panel B (arrow) at higher magnification. C: shows a biocytin filled axon arbor terminating in layer IVβ of galago striate cortex following an injection in the lateral geniculate nucleus. The branches of this arbor are shown at higher magnification in panel D. Scale bars for A,C = 100 μm; for B,D = 10 μm.
beled, appearing as a rich network of bouton-bearing filamentous strands (see Fig. 1C). Typically, a few axon arbor at the sides of the dense bundle could be discriminated clearly from the uninterpretable network of fibers and boutons (Fig. 2).

As mentioned earlier, anterogradely transported biocyatin was best visualized using an Elite ABC kit. Specifically, the staining appeared to be more uniform and the reaction product looked more intense. In one case, we treated alternating sections with Standard and Elite ABC solutions. More labeled axon arbor were found in Elite treated sections, along with portions of arbors in Elite treated sections that could not be matched with any labeled processes (specifically, the remaining half of an arbor) in adjacent sections treated with Standard ABC solution. Increasing the concentration or incubation time of the Standard ABC solution did not provide the same quality of labeling that the Elite kit afforded. Instead, it

Fig. 3. Panel A shows at low magnification labeled cells in the LGN (horizontal section) of the tree shrew, retrogradely labeled by an iontophoretic injection of biocytin into area 17. The larger curved arrow indicates cells shown at higher magnification in panel B, while the smaller, curved arrow indicates cells shown at higher magnification in panel C. OT, optic tract; m, medial; p, posterior. Dotted line marks the lateral and medial borders of the tree shrew lateral geniculate nucleus. Scale for A = 250 μm; for B,C = 40 μm.
increased background staining. It is important to note, however, that background or nonspecific staining tends to increase with Elite kits and is exacerbated by heavy metal intensification. The use of streptavidin-HRP may also provide a solution to this problem, though we have not tried it in primates.

We have been unsuccessful in visualizing anterogradely transported biocytin in 80 and 100 μm thick sections. Biocytin-labeled processes were visualized best in sections 40–60 μm thick. The negative results in thicker sections are most likely the result of limited diffusion.

**Biocytin as a retrograde label for dendrites**

It has been argued that biocytin is a poor, or unreliable, retrograde tracer because cells could only be labeled following enormous (1.5 μl) bulk pressure injections\(^\text{15}\). In this study we compared the results of iontophoretic and pressure methods of injecting biocytin in area 17 of the tree shrew, area 17 of the squirrel monkey, and area 3b in primary somatosensory cortex of the macaque, and find, to the contrary, that biocytin is an excellent retrograde tracer. Both small iontophoretic injections (less than 400 μm in diameter) and larger (0.1–0.3 μl) bulk pressure injections effectively labeled cells. The iontophoretic method appeared to produce well labeled cells more consistently. The cells shown in Fig. 3 were labeled in the LGN of the tree shrew following a small iontophoretic injection in layer 6 of striate cortex (3 s duty cycle, 7 μA, 10 min duration via 30 μm pipette tip). For comparison, the cells shown in Fig. 4 were labeled in VPL of a macaque monkey following a large bulk injection (0.2 μl) in layer 6 of area 3b. In both cases, cells are very well filled, with the dendritic arbor well delineated up to the 3rd and 4th order branches. The staining is clearly superior to that provided by HRP, using identical injection parameters. Only the somata, the primary dendrites, and maybe a fraction of the secondary branches would have been revealed by HRP.

It is important to point out that the cells illustrated in Fig. 3 were photographed in the LGN of a tree shrew that survived 3 days after an iontophoretic biocytin injection into striate cortex, while the cells shown in Fig. 4 were photographed in the VPI of a macaque monkey that only survived 18 h after a bulk pressure injection was made in area 3b. The latter result suggests that retrograde transport of biocytin is rapid. The former result suggests that biocytin is not rapidly degraded once it is transported to its target.

The results of these experiments show that biocytin is a superior alternative to HRP. Retrogradely labeled cells filled with biocytin appear to have been intracellularly

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**Fig. 4.** Panel B shows retrogradely labeled cells in VPL of macaque monkey. These cells were labeled by a large bulk injection of biocytin into area 3b of somatosensory cortex. The open arrow in the central panel indicates a labeled cell, shown at higher magnification in panel A, while the closed arrow indicates another labeled cell shown at higher magnification in panel C. The small curved arrow in panel C points to the labeled cell's axon. Scale bars for A,C = 50 μm; for B = 200 μm.
labeled. Subtle details of fine dendritic appendages, such as dendritic spines and knobs, were discriminated easily. This is best illustrated by the photomicrograph showing pyramidal cells in area 18 of the squirrel monkey filled by a very large (100 μm pipette tip, 7 μA continuous current, 15 min duration) iontophoretic injection of biocytin in area 17 (Fig. 5).

**Multiple labeling with biocytin**

Fig. 6 shows 3 adjacent sections (note the common blood vessel pattern) containing a biocytin injection in tree shrew area 18. The section illustrated in Fig. 6A was treated only for biocytin. This section shows only the outer shell of the injection (limited to layer III) and anterogradely labeled axons and retrogradely labeled cells in layer V. The section illustrated in Fig. 6B was first stained for cytochrome oxidase (CO), then reacted for biocytin. The inset shows a biocytin-labeled branch of an axon arbor located within the CO-stained section. The size of the injection is the same as in Fig. 6A but the density of the staining is increased. Also note that the labeled pyramidal cells and axons in layer V easily can be detected against the CO background. While difficult to illustrate in a black-and-white photograph, biocytin labeled somata and processes are easily discriminated within CO-stained material under the microscope because of differences in contrast. Indeed, boutons, spiny processes, and other fine morphological details are easy to differentiate even at low magnifications (e.g. 25×).

The section shown in Fig. 6C was stained for ChAT immunocytochemistry and reacted for biocytin. Since there are no ChAT + cells in visual cortex, we have shown in

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**Fig. 5.** A: shows retrogradely labeled cells in area 18 of squirrel monkey visual cortex. These cells were labeled by a large iontophoretic injection of biocytin into ipsilateral area 17. The area outlined by the box in panel A is shown at higher magnification in panel B. C: shows an area 18 pyramidal cell (photographed from an adjacent section). Note that the spines along the apical dendrite can be differentiated easily. Scale bar for A = 250 μm; for B = 50 μm; for C = 10 μm.
the inset ChAT + cells labeled in the oculomotor nucleus (located in the midbrain of the same section) as proof that our immunocytochemical protocol stains cells containing the enzyme. As in Fig. 6A, the section in Fig. 6C contains only the peripheral shell of the injection. It is obvious, however, that the injection, and the retrogradely labeled cells in layer V can be discriminated above background. Unlike sections stained for CO and biocytin, or biocytin alone, the biocytin reaction product developed on immunohistochemically treated sections is not black but is a dark red or brown. Here, detection of the biocytin labeled cells is straightforward. However, difficulties could be encountered in differentiating between biocytin and immuno-stained cells located in the same nucleus since the two markers are visualized using the identical chromogen (i.e. DAB) and thus stained processes will show only subtle differences in shades of brown.

DISCUSSION

The major goal of this study was to show that biocytin is an economical, reliable anterograde tracing tool that can be used to visualize axon arbors in detail in several distantly related primate species. Other studies in our laboratory have shown that biocytin also is useful for visualizing axons in adult and neonatal ferrets, suggesting that it may be a useful label for axonal morpholo-

ogy in a variety of species at different ages. If used properly, biocytin should provide investigators who work with mammalian models (other than rodents) the results that the PHA-L method failed to provide. Unlike HRP, biocytin is strictly confined to the immediate site of injection. Therefore, the effective injection site is easy to differentiate. Ultimately, the injection site appears to be limited by the size of the pipette tip. Thus, injections of biocytin can easily be restricted to very small targets such as individual layers or physiologically identifiable zones of a nucleus. Alternatively, larger, very dense injections can label many more axons producing a rich network of labeled terminals in a target(s) similar in density to a TMB reaction product. However, unlike TMB, the biocytin reaction product is stable and not vulnerable to prolonged light exposure or to temporal decay.

This study also showed that biocytin is an excellent retrograde marker, revealing not only the position of individual somata, but also the morphology of their dendrites. The detail revealed by retrograde transport of biocytin far exceeds any image that retrogradely transported HRP could provide using identical injection parameters and survival times. In fact, the details of dendrites revealed in some cells suggest that biocytin can fill the finest processes of cells displaced several centimeters from the injection site after relatively short survival times. The fact that other investigators were unable to demonstrate retrograde filling of dendrites in non-pri-
mate species raises the concern that successful retrograde tracing using biocytin may be useful only in primates. As mentioned earlier, we argue that this is not the case based upon our results in adult and developing ferrets and on unpublished data in cats (Sherman, personal communication).

Finally, this study showed that biocytin can be visualized on sections that have been stained for CO or treated for immunocytochemistry. Previously relationships between HRP labeled cells or axons and CO-dense (or sparse) regions were only inferred by matching blood vessel patterns of serial sections. While findings acquired using the latter technique can be compelling, they are, nevertheless, indirect. With biocytin, relationships can be demonstrated directly. More exciting is the fact that biocytin reaction products can be visualized on sections treated for immunohistochemistry. The only limitation is that, when the same chromogen is used, reaction products may be difficult to differentiate. Two methods could be used to resolve this problem. The first would be to initially react sections to visualize biocytin labeled processes, then intensify the reaction product with metal salts, and finally, process the sections for immunohistochemistry. The second would be simply to use different chromogens to reveal biocytin and the antigen of interest (e.g. alkaline phosphatase, RTTC or FITC).

Co-localization of biocytin labeled products on immunostained sections provides a means to examine more carefully anatomical relationships. For example, it should be possible to examine the morphology of axons terminating on cells of known transmitter type. Since the biocytin product can be visualized with DAB, it should be possible to examine sections at the electron microscopic level. Hence, it should be possible to determine the relationships between biocytin-labeled axonal arbors and immunostained cells (or vice versa). It should also be possible, at the light microscopic level, using a combination of secondary antibodies attached to fluochromes, to actually double-label cells in order to examine the dendritic arborization pattern (as revealed by biocytin) of a cell whose neurotransmitter type was identified immunocytochemically.

As with other methods, biocytin has shortcomings. Biocytin appears to have a short-shelf-life even when stored as a powder at the recommended temperature of −20 °C. Therefore, we recommend that investigators buy small portions of biocytin (relatively inexpensive at this writing), shortly before experiments are to be completed.

A second limitation of biocytin is that it does not appear to be transported from the eye. We have attempted to fill retinogeniculate arbor either by iontophoretically injecting a 3–10% solution of biocytin into the retina of adult or developing tree shrews, or by pressure injecting biocytin (0.3 μl) into the vitreous chamber of adult galagos and adult marmosets (Callithrix jacchus). In each case, the vitreous body congealed and the orbit collapsed within 24 h. No neural processes were observed in the LGN, and there was no evidence of labeled retinal ganglion cells from the fragments of retina that we were able to salvage. Recently, however, we have been able to label some axons from the eye with a biotin ester using a procedure described by Hafer.

Short-comings are not unique to biocytin. Once reconstituted PHA-L also is very short-lived. Vector Laboratories claims 6 months (at −20 °C), but this estimate is probably very generous. Moreover, neither HRP nor PHA-L appears to fill axons in detail after being injected into the eye.

In conclusion, we have shown that biocytin is an excellent tracer that may be used to study the detailed morphological features of cell bodies, axons, and dendrites in primates. Injected extracellularly, it is transported in either the anterograde or retrograde direction to completely label processes as though they were filled by intracellular injection. Biocytin is also far more economical and reliable to use than PHA-L. The fact that biocytin labeled processes may be visualized on CO- and immunostained sections should allow investigators to make direct comparisons that are not possible with other labels.

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