Loose-patch–juxtacellular recording in vivo—A method for functional characterization and labeling of neurons in macaque V1

S. Joshi*, M.J. Hawken

Center for Neural Science, New York University, 4 Washington Place, Rm 809, New York, NY 10003, United States

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Abstract

We describe a method that uses a modified version of juxtacellular labeling [Pinault D. A novel single-cell staining procedure performed in vivo under electrophysiological control: morpho-functional features of juxtacellularly labeled thalamic cells and other central neurons with biocytin or neurobiotin. J Neurosci Meth 1996;65:113–36], which allows us to functionally characterize and subsequently label single neurons in vivo in macaque V1. The method is generally applicable in acute in vivo preparations. Extracellular recording is made with a patch electrode when the electrode is attached to the cell membrane. Initially a ‘blind’ search method is used as a guide to obtaining a cell attached configuration that we refer to as a loose-patch (LP). The neuron’s receptive field properties are functionally characterized, the neuron is labeled and then characterization is confirmed, all in the LP configuration. There are a number of advantages of the method that we describe over other methods. First, we have found that we can obtain stable extracellular recordings for periods of hours that enable us to make a relatively comprehensive visual functional characterization of a neuron’s receptive field properties. Second, because the electrode is closely apposed to the cell we obtain excellent isolation of the extracellular spike. Third, the method provides labeling that gives complete dendritic and axonal filling that survives over a number of days, which is an important feature in acute primate experiments. Fourth, the in vivo method of labeling and reconstructing neurons gives complete three-dimensional structure of the neuron including its intra-cortical axonal arbor. These features overcome known limits of the established methods of studying neuronal morphology including the Golgi stain (limited when adult tissue is used) and in vitro whole cell methods (incomplete axonal filling due to limited slice thickness). They also overcome the known limits of the established method of combined function-morphology studies i.e. intracellular recording in vivo. The modified juxtacellular method provides a reliable alternative to the difficult method of characterization by extracellular recording and subsequent intracellular labeling [Anderson JC, Martin KAC, Whitteridge D. Form, function and intracortical projections of neurons in the striate cortex of the monkey Macacus nemestrinus. Cerebral Cortex 1993;3:412–20]. We show the method can be used to record at a range of depths through V1 cortex allowing for sampling of neurons in the different layers and functional subpopulations. Links can then be made with existing knowledge about the anatomical organization of V1, the various morphological classes of neurons found therein, their functional connectivity and visual response properties.

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1. Introduction

Four main methods have been described to record from neurons to obtain a functional characterization followed by labeling and subsequent morphological identification of the neuronal type and its dendritic and axonal arbors. In the case of neurons in the visual pathway, functional characterization implies a receptive field characterization. Two of these methods use intracellular recording for the whole duration (of recording and labeling), either with sharp electrodes [Azouz et al., 1997; Cardin et al., 2005; Gilbert and Wiesel, 1979; Nowak et al., 2003; Van Essen and Kelly, 1973] or with whole-cell patch electrodes [Hirsch et al., 1995, 1998, 2002, 2003]. In the third method, using sharp electrodes, the initial recording is made extracellularly while the neuron’s receptive field is functionally characterized. The electrode is then advanced to obtain an intracellular configuration and labeling is performed [Anderson et al., 1993; Martin and


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* Corresponding author. Tel.: +1 212 998 7613; fax: +1 212 995 4860.

E-mail addresses: siddha@cns.nyu.edu (S. Joshi), mjh@cns.nyu.edu (M.J. Hawken).

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Whitteridge, 1984a, 1984b). In this method, the receptive field preferences are verified in the intracelllar stage. The fourth method is termed juxtacellular recording (Pinault, 1996). In this method a sharp electrode is used to obtain extracellular recording and then moved to be in close apposition to the cell membrane where labeling is obtained by applying a train of current pulses (Pinault, 1996). None of these methods have been widely used in studies of the macaque visual pathway perhaps because the soma sizes are relatively small compared with other species, thus making long stable recordings difficult.

Following the pioneering work of Hubel and Wiesel (1962, 1968), a wide range of studies using extracellular recording in primate V1 have identified numerous properties of receptive fields that can be used to separate functional classes of cells. These properties include color (Dow and Gouras, 1973; Livingstone and Hubel, 1984), spatial frequency (Schiller et al., 1976) and direction selectivity (Orban et al., 1986; Poggio et al., 1985). Quantitative assessment of neuronal firing has refined and extended the range of properties that can be used to define receptive fields. Such properties include spatial frequency (De Valois et al., 1982; Xing et al., 2004), direction selectivity (Hawken et al., 1988), color (Johnson et al., 2001; Lennie et al., 1990), dynamics of orientation tuning (Ringach et al., 1997), stimulus size (Cavanaugh et al., 2002; Levitt and Lund, 1997; Sceniak et al., 1999) and binocular disparity (Cumming and Parker, 1997). Furthermore, recent developments in modeling of the properties of visual neurons make specific predictions about the tuning, dynamics and firing patterns of different neuronal types (McLaughlin et al., 2000; Miller, 2003; Shelley et al., 2002; Troyer et al., 1998).

The morphology of V1 neurons has been studied extensively using a number of methods. Such studies provide reconstructions of neurons and their laminar locations. The labeled neurons are obtained from Golgi stained sections of monkey visual cortex (Lund, 1973, 1987; Lund et al., 1975, 1988; Lund and Wu, 1997; Somogyi et al., 1981) as well as from intracellular fills of neurons both in vivo in macaque striate cortex (Anderson et al., 1993) and cat striate cortex (Gilbert and Wiesel, 1979; Hirsch et al., 1995, 1998, 2002, 2003; Martin et al., 1983; Martin and Whitteridge, 1984a, 1984b; Van Essen and Kelly, 1973) and in vitro in slices of monkey V1 (Katz et al., 1989; Wiser and Callaway, 1996) as well as in sections of fixed tissue (Elston et al., 1999).

Thus, while there is substantial data on the functional properties of macaque V1 neurons on the one hand and on their morphology on the other, there is a relative lack of data that directly relates the two. Although the four methods discussed above can potentially bridge this divide there are limitations on the amount of functional characterization that can be obtained during the period of stable recording, and such experiments have almost exclusively been done successfully in cat striate cortex. In order to relate the properties of identified neurons either to classes of neurons from quantitative extracellular studies or to model neurons we need to obtain measurements of the quantitative properties of their receptive fields along a number of stimulus dimensions as well as the morphological properties. To achieve this goal in the primate V1 we have developed a method that incorporates a number of features from previous methods with the cell-attached recording technique. We call this hybrid method loose-patch–juxtacellular (LP–JC) recording. A major advantage of an in vivo method of characterizing and labeling single neurons is that the complete three-dimensional structure of the neuron and, potentially, its intra-cortical axonal projection, is recovered. Reconstruction of neurons labeled in vitro is limited by the fact that the slice preparations used are typically about 300 μm thick and fibers extending beyond this distance out of the thickness of the sections are lost. With an in vivo method, it may be possible to recover the projections of labeled neurons to their cortical or subcortical targets.

Pinault (1996) described a method of extracellular recording with glass electrodes that could be used to also label a recorded cell. Here, we describe a method that uses a modified version of juxtacellular labeling (Pinault, 1996), which allows us to functionally characterize and subsequently label single neurons in vivo in macaque V1. There are a number of advantages of the method that we describe over other in vivo methods such as whole-cell and sharp electrode intracellular recording. First, using electrodes with tip diameters of 1–2 μm and impedances between 5 and 15 MΩ (1 kHz; patch electrodes), we have found that we can obtain stable extracellular recordings over periods of hours that enable us to make a relatively comprehensive visual functional characterization of a neuron’s receptive field properties. This is an advantage over intracellular methods where retaining a stable recording in cortex for hours in vivo is unusual. Second, because the electrode is closely apposed to the cell we obtain excellent isolation of the extracellular spike. Third, the method provides labeling that gives complete dendritic and axonal filling that survives over a number of days, which is an important feature in acute primate experiments.

2. Materials and methods

Initially, we performed a series of preliminary experiments in slices of cortex in vitro. There were three main aims of the in vitro experiments: first, to select the range of electrode tip sizes that did not penetrate the neuron in loose-patch configuration yet showed satisfactory recording of the extracellular action potentials; second, to establish the characteristic changes in electrode impedance when making a ‘blind loose-patch’ in vivo and to establish initial electrode pressure parameters to obtain loose-patch recordings that do not penetrate the cell membrane while keeping the tip clear; third, to determine the appropriate range of current pulse amplitude and labeling duration to obtain labeling with biocytin (Sigma–Aldrich) and BDA (biotinylated dextran amine; Molecular Probes) and to compare this labeling with that obtained in whole-cell mode. We then used these parameters as a guide for in vivo experiments in the anesthetized paralyzed macaque. The in vitro experiments are described first.

2.1. In vitro experiments

The first and second aims were to choose electrodes and develop a protocol to achieve the loose-patch (cell-attached) configuration. With traditional metal extracellular electrodes (typically in our laboratory—glass-coated tungsten (Merrill and
vivo in the absence of visual guidance. We used 300 μm slices of rat somatosensory cortex. (B) Current response to voltage steps; arrow indicates decrease in current with proximity to cell membrane.

Ainsworth, 1972)), it is possible to search for cells while providing visual stimuli since modulation of the local population (hash) is clearly audible. Small movements of the metal electrode generally increase the size of the extracellular action potential until a single spike can be discriminated from the background hash. The glass electrodes that we used did not provide such a background signal, nor did the spike begin as part of the hash and emerge when the electrode got closer to the cell. Thus, we needed to find a means of monitoring when the electrode was approaching a cell. Under visual guidance (infra-red differential interference microscopy, IR-DIC), in vitro (Fig. 1A), we developed a technique based on changes in the electrode impedance as it neared a cell membrane that would allow us to loose-patch neurons in vivo in the absence of visual guidance. We used 300 μm slices from rats (age P10–P30) and patch electrodes of impedance ranging from 1 to 2.5 MΩ. The tip diameters of these electrodes ranged from 1 to 2.5 μm. For details of slice preparation and maintenance, see Oviedo and Reyes (2002). A Dagan BVC-700 intracellular amplifier was used for recording voltage and current signals and for generating voltage commands or current pulses. A Goldstar FG-8002 function generator provided a square wave pulse with the required duty cycle (200 ms ON, 200 ms OFF) as a TTL signal to the GATE input of the Dagan amplifier. The current amplitude could then be set from the amplifier when in current clamp (I_{clamp}) mode.

In voltage-clamp mode (V_{clamp}) the holding voltage was set at the break-in value of about −65 mV while a step of 10–13 mV was applied at ~10 Hz (Fig. 1B). Once the electrode was positioned close to a cell, the current response to this applied voltage was monitored on the amplifier display as well as on an oscilloscope. A positive pressure of about 20–30 mmHg was maintained in the patch pipette to keep the tip clear. Pressure was applied using a 3 cc syringe connected to the patch pipette holder via a length of tubing. A digital pressure gauge (Digitman, Netech) was used to monitor the amount of pressure or suction applied. In each slice, we attempted to patch and label one neuron in whole-cell configuration (intracellular) and then loose-patch (LP) one or more additional neurons with the same electrode so that we could compare whole-cell and LP labeling.

As mentioned above, these in vitro experiments were done under visual guidance (IR-DIC). We noticed that when the electrode is moved very close to a cell, this pressure causes a small patch of membrane to be pushed away from the tip; this is visible as a dimple on the surface of the cell. Due to the close apposition of the electrode tip with the membrane, this is also registered as an increase in the tip resistance. For loose-patching, positive pressure was released and suction applied (usually −10 to −15 mmHg) until the tip resistance increased by a factor of three to ten times the starting value. We observed that releasing positive pressure and applying suction (negative pressure) causes the local region of the cell membrane to be sucked into the micropipette tip, without detaching the membrane, causing this further increase in the tip resistance. Locking the suction at this value causes the seal to be maintained for the duration of the recording. This was designated as a loose-patch (LP) configuration. In this configuration we could record large amplitude extracellular action potentials, see Fig. 3P for an example of the extracellular action potentials recorded in vivo with the same electrode configuration. These are the steps that we observed in the in vitro experiments and that we applied to the in vivo experiments.

The third aim was to determine the appropriate range of current amplitudes and labeling duration in order to obtain good fills and to compare these with intracellular fills obtained in the same slice of cortex. Once a loose-patch configuration was obtained, we applied current pulses of 0.3–3 nA for 200 ms ON and 200 ms OFF following Pinault (1996) for up to 20 min. At the end of a recording period (1–5 h) the slice was fixed overnight in 4% paraformaldehyde, or in other cases the slice was fixed by immersing in 4% paraformaldehyde and 0.25–0.5% glutaraldehyde and microwaved at high power for 5 s. The microwave fixation procedure is commonly followed when tissue from in vitro physiology experiments requires processing for electron microscopy (R. Levy, personal communication). Following fixation the slice was reacted with standard Vector ABC kits. Biocytin is progressively degraded in vivo during
the post-labeling survival period so we also tested the proto-
col using tracers other than biocytin. Pinault (1996) suggested
using biotin–dextran conjugate markers when long (>48 h) post-
labeling survival times were anticipated. BDA is widely used in
tract tracing experiments that require the axonal transport of
tracer over long distances—a process that can take several days.
As typical in vivo experiments last up to 5 days, we wanted to
ensure that the protocol would work with such markers. In vitro
experiments were conducted with BDA to determine whether
the same range of current amplitudes and durations gave satisfac-
tory labeling.

2.2. In vivo experiments

The main objective of our study was to develop a proto-
col, guided by the results of the in vitro experiments, to make
loose-patch recordings from macaque V1 neurons, obtain visual
functional characterization for and then to label the recorded
neurons. Acute experiments were performed on adult Old-World
monkeys (Macaca fascicularis, Macaca radiata and Macaca
mulatta) weighing between 1.5 and 10 kg, in strict compliance
with NIH and institutional guidelines for the care and experi-
mental use of animals.

2.2.1. Preparation

Animals were prepared for recording as described else-
where (Xing et al., 2004). Briefly, the monkey was tranquil-
ized with diazepam (0.5 mg/kg, i.m.), anesthesia was induced
with ketamine (5–20 mg/kg, i.m.), then maintained with isoflu-
orane (1.5–3.5% in air) during surgery. At the initial stage of
surgery atropine (0.1 mg/kg, i.p.) was given to suppress mucus
secretion; dexamethasone (0.5 mg/kg, i.m.) administered to pre-
vent cerebral edema; and a broad-spectrum antibiotic, bicillin
(50,000 i.u./kg, i.m.) was injected to prevent infection. Both
femoral veins were cannulated for the delivery of drugs for the
remainder of the experiment, and a tracheal tube was inserted
for artificial respiration. The animal was placed in a stereotaxic
apparatus, and attached to physiological monitors. Heart rate,
EKG, blood pressure, and expired CO2 level were monitored
continuously. Core body temperature was monitored by a rec-
tal probe and the animal’s body temperature was maintained at
37 °C with a heating pad. At this point, the animal was adminis-
tered an opiate anesthetic (sufentanil citrate, 6–18 µg/kg/h i.v.)
and attached to an artificial respirator. EEG leads were attached
to the skull above the frontal bone, differentially amplified and
monitored continuously on an oscilloscope. The EEG was dom-
inated by slow-wave high-amplitude components indicative of
anesthesia. Once the surgery was completed, muscle paralysis
was induced and maintained with vecuronium bromide (Nor-
curon, 0.1 mg/kg/h i.v.) and anesthesia was continued with con-
tinuous sufentanil infusion.

2.2.2. Optics

Both pupils were dilated with topical administration of 1%
atropine sulfate and immediately protected with gas perme-
able contact lenses with ophthalmic antibiotic (gentamicin). The
eyes were regularly checked throughout the experiment, and
the lenses and cornea were rinsed when necessary. External
lenses were used to correctly focus the display monitor image
on the retina. This correction was initially estimated with an
ophthalmoscope, and later checked and corrected if necessary
by optimizing the responsiveness of spike activity for high spa-
tial frequency stimulation. Both foveas were mapped using a
reversible ophthalmoscope onto a tangent screen, so that recep-
tive fields could also be mapped in relation to the foveal position.

2.2.3. Electrode penetrations

A grid of 10–12 small (~2.5 mm across with adjacent edges
separated by at least 2 mm) craniotomies was made posterior
to the lunate sulcus, over one hemisphere. The separation between
craniotomies was maintained to prevent any ambiguity in the
identification of the penetrations and assignment of cells to
particular tracks and to maintain integrity of the dura. In each
craniotomy the skull was first thinned down until the dura was
just visible. A 27-gauge needle was used to carefully make an
incision in the dura and, with its tip bent, used to fold back a
small dural flap resulting in a small durotomy, minimally suffi-
cient for the electrode to enter (approximately 0.1–0.3 mm) and
also to ensure that the surface at the point of entry was free from
blood vessels. The electrode was introduced into cortex under
visual guidance using a Zeiss operating microscope. Pressure
(10–30 mmHg) was maintained in the electrode to keep the tip
clear. As in the in vitro experiments, pressure was applied using
a 3 cc syringe connected to the patch pipette holder via a length
of tubing. A digital pressure gauge (Digimano, Netech) was used
to monitor the amount of pressure or suction applied. We found
that using higher pressures could result in cortical tissue damage
as well as excessive ejection of tracer-containing electrode solu-
tion. Excessive tracer ejection can result in intense background
staining and labeling of neurons—making it impossible to iden-
tify the targeted neuron or to find out if the targeted neuron was
labeled in the first place. The electrode impedance was measured
as soon as it entered the CSF and was monitored at all stages of
the experiment. The electrode was advanced rapidly into cortex,
using a motorized stepping microdrive (Narishige), to a depth
of 400–700 μm. At this point the electrode was allowed to equi-
brate for 10–20 min.

After this period of waiting, the electrode was advanced in
steps of 1 μm. The amplifier was in voltage clamp (Vclamp) mode,
and as described for the in vitro experiments, the tip resistance
was monitored by observing the current response to steps of volt-
age. Guided by the in vitro experiments, the holding voltage was
set at −65 mV and a voltage step of about −13 mV applied at
10 Hz. This last step allows for visual monitoring of the tip resis-
tance (see Fig. 1B). It should be noted that the holding voltage is
typically set at about −65 mV in experiments when an intra-
cellular recording is to be attempted. This is done so as to avoid
introducing a large voltage drop at the electrode tip where the
patch is made. Thus it is not an essential step and we have found
it sufficient to apply a small voltage step to monitor the tip resis-
tance. The electrodes were patch pipettes with typical impedance
in the range 5–15 MΩ (1 kHz) and tip diameter of 1–2.5 μm. This
made them unsuitable for searching for cells in voltage recording
mode because the amplitude of the extracellular action poten-
tial was very small unless the electrode was in contact with the neuronal membrane. As the electrode was advanced we used a large drop in the amplitude of the current to the voltage pulse as an indication that the electrode was approaching a cell membrane. This was monitored on the amplifier readout (as current in nanoamperes) as well as visually on an oscilloscope. An example from the in vitro experiments showing this increase in resistance can be seen in Fig. 1B. After a change in current amplitude of at least 50%, pressure was released and a small amount of suction applied (usually 10–15 mmHg). If this resulted in an increase in tip resistance by a factor of at least three to ten times the starting value, we stopped applying suction and maintained suction at this value. We did not exceed 25 mmHg negative pressure, so if a seal was not obtained we released suction and continued searching. For those occasions where we obtained a seal we switched to $I_{\text{clamp}}$ mode to ascertain that the electrode was in fact in contact with a neuron and to begin recording extracellular spikes; we termed this a loose-patch–juxtacellular (LP–JC) configuration. Sometimes cells were silent until stimulated visually. In this cell-attached mode, recorded action potential amplitudes were around 5 mV. Occasionally, stability was poor when the first cell in a penetration was isolated. In such cases, a small amount of agar was poured into the craniotomy. This usually resulted in improved stability.

2.2.4. Visual characterization

In the experiments described here our objective was to show that we could obtain a quantitative receptive field characterization using a set of stimuli that enable visual functional properties to be measured. Once the cell was isolated, the orientation, size preference and ocular dominance were quickly estimated by using stimuli generated on a tangent screen. The non-dominant eye was then occluded and all subsequent visual stimulation was through the dominant eye. We then made quantitative estimates of tuning using computer generated sinewave grating stimuli to obtain tuning curves for orientation, spatial frequency, temporal frequency, contrast, size, cone-isolating spatial frequency and color-exchange responses (Johnson et al., 2004). For details of stimulus generation and data acquisition, see Xing et al. (2004). Once basic visual tuning was obtained, we began the labeling protocol.

2.2.5. In vivo labeling

Continuous monitoring of the spike waveform allowed us to monitor the health of the cell at all times. We did this in two ways—on the computer used for discriminating and sorting spikes (Johnson et al., 2004) and on a second computer running Spike2 software via a CED 1401 interface (Cambridge Electronic Design). We digitized the voltage records at 10 kHz and stored them. Once tuning curves were recorded, we began the labeling protocol described in Section 2.1. One important difference between the in vitro protocol and the in vivo protocol was in the current amplitude (also see Sections 3 and 4 for a discussion of the difference between the current levels required in vivo and in vitro). We began the in vivo labeling protocol with a small current amplitude (about 0.1 nA) and increased this while monitoring the spike waveform. We lowered the amplitude if the labeling current induced a continuous train of action potentials in response to the current pulses. Typically, the current range was between 0.2 and 2 nA and the current was applied for 10–20 min. Once this protocol was run, we repeated the quantitative visual characterization of the neuron. We consider this step important as it provides an additional safeguard to ensure that the same cell was being recorded before, during and after labeling. While recording, it is possible for the electrode to move relative to the neuron thus resulting in the characterization of one neuron and the labeling of a different neuron. That this is a possibility was learned in two early in vivo trial experiments (in which we did not re-characterize after labeling) that resulted in pairs of cells being labeled.

To obtain the most reliable correlation between the functional and morphological characterization we consider two steps to be important. First, monitor the isolation of the neuron throughout the recording and labeling procedure. Second, confirm the functional characterization by at least a partial re-characterization of the visual responses of the neuron after labeling. If these steps are followed we consider that the tuning curves can be related to a particular labeled cell with confidence.

Once the recording and labeling was complete, suction was released and the electrode withdrawn slowly in steps of 1 μm for at least 20 μm. This was done to minimize damage to the cell. We have noticed in our in vitro experiments, in whole-cell as well as cell-attached configurations, that pulling away rapidly from the neuron causes the membrane to tear away with the electrode often killing the cell. The durotomy was covered with a small piece of gelfoam and sealed with bonewax before moving on to the next craniotomy.

Experiments were terminated by i.v. injection of a lethal dose of pentobarbital (60 mg/kg), after which the animal was quickly perfused through the heart with 1 liter of heparinized saline (0.01 M phosphate buffered saline, pH 7.4, PBS) followed by 2–3 liters of fixative (4% paraformaldehyde, 0.25% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4). Following perfusion, occipital cortex blocks were prepared by making blocking cuts normal to the surface and along rows of penetrations—identified by durotomary marks. The block was then glued onto the vibratome stage on its medial or lateral surface (whichever was larger). The tissue sections were thus sagittal near the blocked surface closest to the rows of penetrations used to align the blocking knife, and parasagittal thereafter. Tissue was sectioned in PB at 50–70 μm on a Leica vibratome and stored in sequence in PB in single wells of cell culture dishes. (In some cases, the tissue was rinsed twice before transferring with NaBH₄—were rinsed until all bubbles disappeared). The solution was prepared using standard Vector Elite ABC kits. The sections were immersed in a solution of 0.3% H₂O₂ and 8% methanol in PB for 10 min. The sections were then rinsed in PB (3×10 min; or when treated with NaBH₄—were rinsed until all bubbles disappeared). The tissue was then treated for endogeneous peroxidase activity by immersing in a solution of 0.3% H₂O₂ and 8% methanol in PB for 30 min. The sections were rinsed in PB (3×10 min). ABC solution was prepared using standard Vector Elite ABC kits. The solution was prepared in 0.01 M phosphate buffered saline (PBS, pH 7.4) with 0.05–0.1% Triton. All sections were incubated in the ABC solution overnight (12–16 h). The next day,
they were rinsed in PBS (2 × 10 min; 1 × 30 min) and reacted using DAB (11.5 mg per 50 ml PBS) and hydrogen peroxide (5 μl/50 ml DAB solution). The reaction time was judged by observing tissue by eye or under a microscope and was typically 2–10 min (occasionally longer—up to 20 min). Following the DAB reaction, sections were rinsed in PBS (3 ×) before examination. We first examined the wet sections at 4× to 20× to try and locate the labeled neurons. We then prepared every third section for the cytochrome oxidase (CO) reaction taking care to ensure that the sections thus selected did not contain a labeled neuron’s cell body. Following the CO reaction, sections were rinsed in PBS (3 ×). The sections were then mounted on subbed slides and dried overnight. The following day they were dehydrated in a graded series of alcohols of increasing strength, defatted (in xylene) and coverslipped.

Initially, sections were examined and reference tracings made for sections in which electrode tracks or labeled cells were found. When labeled neurons were recovered, we determined the location of the entry point relative to the lunate sulcus as well as the position relative to adjacent tracks to verify the position of that track in the grid. The distance of the labeled neuron from the entry point was also measured and compared with the microdrive reading. Once labeled neurons were located and identified, higher magnification reference tracings were made with two objectives—to identify which sections would need to be traced on a computer tracing program and to identify and comment on morphological features of interest. We then used a computer-tracing program (Neurolucida, Microbrightfield) to make a three-dimensional tracing of each neuron.

3. Results

3.1. In vitro experiments

As described in Section 2, we labeled a number of cells in vitro. In each slice of cortex (somatosensory, often including hippocampus) we labeled one neuron intracellularly and at least one or more by the LP–JC protocol. This provided a means of comparing the labeling obtained by the two methods in a single cortical slice. A neuron labeled by the LP–JC method is shown in Fig. 2A. The quality of the fill attainable by this method can be compared with the intracellular fill shown in Fig. 2B. In both, detailed morphological features including extensive dendritic branching and spines are clearly visible; additionally background labeling is low (Fig. 2C and D). The axon of the neuron shown in Fig. 2A was not conserved in the slice preparation as it left the plane of the slice a short distance from the axon hillock. An example of an axonal arbor from a different LP–JC labeled neuron is shown in Fig. 2E.

We loose-patched and ran the LP–JC labeling protocol on 28 cells (Table 1). Of these, the quality of labeling of 14 cells was equivalent to that obtained for the intracellular fills. Another seven cells were less well labeled although the staining allowed for identification of the neuronal morphology. The quality of labeling did not depend on the range of currents used. The remaining seven labeling attempts resulted in the labeling of a region of the neuropil rather than a single neuron and these were classified as failed attempts. An example of such an attempt is shown in Fig. 2F.

In another set of in vitro experiments we tested the use of electrode solutions other than the one described in Section 2.
The juxtacellular method described by Pinault (1996) made use of higher impedance (25–70 Ω) electrodes filled with neurotobin in a solution of saline or potassium acetate. We wanted to test whether these solutions could also be used with our method. This was unsuccessful and we would not recommend these electrode solutions when used with our LP–JC protocol for recording and labeling. The tip geometry of patch electrodes is quite different from that of sharp electrodes and it is this geometry that, in part, determines the impedance of these electrodes. The lower impedance and larger tip opening of our patch electrodes may have resulted in relatively large volumes of the saline (or acetate) solution leaking out in close proximity to, or in contact with a neurons membrane, thus compromising the health of the cell. Such leakage of electrode solution is less likely to be an issue with the higher impedance electrodes used in true juxtacellular recording (or sharp intracellular recording) where such solutions are used.

3.2. In vivo experiments

We loose-patched 25 V1 neurons that we subsequently ran the complete LP–JC protocol on – i.e. – characterize, label and re-characterize. Of these, we recovered 12 well-labeled neurons (Table 2, row 1). In another seven attempts, isolation of functionally characterized neurons was lost during the labeling phase. Two of these neurons were subsequently recovered (Table 2, row 2). In two further attempts, two well-labeled pairs of neurons were recovered (Table 2, row 3). For all the recovered neurons we used low labeling currents (0.3–0.5 nA; Table 2, rows 1 and 2). This range of currents is considerably lower than that used by Pinault (1996) and used subsequently by others (Klausberger et al., 2003, 2005). Each time we used higher currents to label a neuron after the initial characterization phase of the protocol, the neuron was lost during the labeling phase. All eight neurons (Table 2, row 4) that we carried out the initial characterization for and then attempted to label with 1–2 nA current were lost during the labeling procedure. High currents (1 nA or greater) could result in successful labeling of neurons if only labeling – but not characterizing – the neuron was attempted. We found out that labeling is possible with high currents in an early in vivo experiment where we did not attempt to characterize cells—only label them with 2 nA current (Table 2, row 5). It is possible that staying attached to the neuron for the period of the initial visual characterization results in local electroporation of the membrane so that subsequently lower currents are required to label the cell.

We present the morphology and functional properties of two characterized and labeled neurons, the first a layer 5 pyramidal neuron (Fig. 3) and the second a layer 4Cα spiny stellate neuron (Fig. 5). Further, as another example of the extent of axonal filling achieved by our method, we present the computer reconstruction of a layer 4B star-pyramidal neuron (Fig. 6). All three neurons were labeled with BDA.

3.2.1. Layer 5 pyramidal neuron—morphology

One example of a fully characterized and labeled neuron is shown in Fig. 3. This neuron was recovered 33 h after labeling. The morphology of the cell body and the main dendritic branches are characteristic of a pyramidal cell. The cell body is in layer 5 (Fig. 3A, E, F) with a large apical dendrite that rises for about 1 mm to layer 1 (Fig. 3F, right). The pattern of basal dendritic branching is mainly confined to a lateral spread in lower layer 5 and the upper part of layer 6, extending up to 300 μm or more from the cell body (Fig. 3E). There is an additional set of branches that do not spread so far laterally but ascend into upper layer 5 and lower layer 4CB and descend into layer 6 (Fig. 3E). The lateral spread of these branches is about 100 μm. We made a 3D reconstruction of the dendritic branches through a thickness of over 0.5 mm (measured lateral–medial). When viewed in a horizontal projection, i.e. from above (Fig. 3H), a pronounced dendritic asymmetry is visible; there is a major bilateral elongation along the 135°–315° axis.

The quality of the fill is revealed in the photomicrographs shown in Fig. 3B–D and in the reconstructions shown in Fig. 3E–H. Overall the fills matched well with cells stained intracellularly in vivo in cat and rat (see, for example, reconstructions shown in Hirsch et al., 1998). Fine dendritic branches were visible along with spines and fine axonal branches and thickening are visible under high magnification. In addition, the axon is clearly visible (Fig. 3B) as it enters the white matter (350 μm from the axon hillock) suggesting that this might belong to a class of layer 5 neurons that project to subcortical nuclei such as the superior colliculus or the pulvinar, or to both (Lund et al., 1975). Hubener et al. (1990) reported a class of spine free pyramidal corticocortical cells in layer 5 of cat striate cortex. They found that these have fewer axon collaterals and that these do not arborize in layer 6. As seen in Fig. 3F, the intracortical branch of our example neurons’ axon arborizes locally, mainly within layer 5; the local spread is about 300 μm horizontally (Fig. 3F and G, which shows a horizontal projection of the axon tracing).

Extensive axonal branching is often found that extends millimeters in some cortical neurons (Gilbert and Wiesel, 1983; Hirsch et al., 1998). Neurons labeled in vitro can only provide information about dendritic branching and axonal arborization

<table>
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<th>Time (min)</th>
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All recordings loose-patch–juxtacellular (LP–JC); L: isolation lost during labeling.
Fig. 3. Results from in vitro experiments: a BDA labeled layer 5 neuron. (A) Low power (5×) image of the neuron showing its laminar position. (B) Montage of images at 40×. The axon (labeled ‘A’) is seen descending into the white matter (WM). (C) High power (100×) micrograph of the apical dendrite shows aspiny branches. (D) High power micrograph (100×) of axon hillock. (E–H) Computer assisted reconstruction of this neuron. (E) Front view of soma and dendritic arbor. (F) Axon (left) and dendrite (right); arrow indicates continuing WM branch. (G) Top view of axonal arbor; the arrow shows the projecting (WM) branch of the axon. (H) Top view of the dendritic tree; circle diameter = 400 μm. Anatomical directions shown are for G and H only. This neuron was drawn from nine sections of thickness 60 μm (540 μm block of cortex, parasagittal plane); the axon coursing through the WM (not shown) was drawn from 39 sections (2.340 mm block of cortex, parasagittal plane). This example is from one of our earliest experiments when we had not begun staining alternate sections (or every third section) for cytochrome oxidase. We have found that the background resulting from the ABC–DAB reaction in macaque tissue allows for the identification of the 4C–5, 5–6 and 4B–4C boundaries. In later experiments (for all of the other cells shown in the paper), we have confirmed this by identifying boundaries in adjacent sections, all reacted by the ABC–DAB method with alternate sections stained for cytochrome oxidase. The identified boundaries are in excellent agreement. It may be noted that the boundaries that cannot be easily identified by the ABC–DAB background are the 4A–4B and 2/3–4A boundaries. (I–O) These panels show the characterization of the functional properties of this neuron: orientation tuning (I), temporal frequency tuning (J), spatial frequency tuning at two drift directions at the preferred orientation (K and L), Contrast response (M), area tuning (N) and color exchange (O); M, N and O obtained before labeling. (P) Extracellular traces recorded immediately after loose-patch formation (upper left); during a break in current injection and while stimulating visually (upper middle); after the current injection protocol was complete (upper right). Arrows in the middle trace indicate the beginning of a stimulation sequence. (P; lower panel) Three spike waveforms selected from each phase of the protocol. The tuning obtained before labeling (solid lines) is very similar to that obtained after labeling (dashed lines). There is an increase in spontaneous discharge rate after labeling (compare solid and dashed horizontal lines in I–L) which typically goes back to pre-labeling levels 15–20 min after the labeling sequence is completed.

Through the thickness of the slice (typically 300–400 μm)—a technical limitation.

This is also exemplified in the neuron (labeled in vitro) shown in Fig. 2A, where the axon was not conserved in the slice preparation. Axonal labeling that we have obtained in cortical neurons shows filled fine branches millimeters from the cell body. These branches can be traced horizontally within a single parasagittal section as well as through many sections to give a full three-dimensional profile. Fig. 3F and G shows reconstructions of the axonal projections that extend outside the region of the
dendritic arbor. The parasagittal projection (Fig. 3F) shows a horizontal spread along the layer 5/6 border that extends for about 600 μm and another branch that rises to layer 4C. We were also able to trace more than 4 mm of the projecting axon (indicated by arrow in Fig. 3F and G) into the white matter through about 2.3 mm of thickness (in the lateral–medial axis). The axonal spread revealed by such labeling compares well with that obtained by intracellular fills of neurons (Hirsch et al., 1998; Wiser and Callaway, 1996).

3.2.2. Layer 5 pyramidal neuron—functional characterization

The tuning curves for this cell, obtained before the labeling protocol was run, are shown in Fig. 3I–O (solid lines). It responded robustly above the spontaneous rate (solid horizontal line shows the spontaneous firing rate before labeling) to visual stimulation with peak firing rates of 60 spikes/s at the preferred orientation (Fig. 3I–O). The response was an elevation in discharge (rather than a modulation to the passage of the grating), which is a characteristic of complex cells (Skottun et al., 1991). It showed sharp tuning in orientation (Fig. 3I) and was band-pass in spatial frequency (Fig. 3K and L) with a peak at about 2 cycles/°. The cell was low-pass in temporal frequency (Fig. 3J). Functional tuning properties after the 20 min of labeling were almost identical to those before labeling as shown by the dashed curves in Fig. 3I–L. There was a change in the spontaneous discharge rate of the cell after labeling around 10 spikes/s to 20 spikes/s (compare the horizontal solid and dashed lines in Fig. 3I–L). It was a relatively common observation among the cells that we held after running the labeling protocol there was an increase in the spontaneous discharge. For cells that we held for an hour or more after labeling, the spontaneous returned to the pre-labeling levels within 15–20 min. Further illustration of the range of visual functional characterization that we routinely obtained is exemplified by the tuning curves shown in Fig. 3M–O. For this neuron we also obtained the contrast response function (Fig. 3M), area (size) tuning (Fig. 3N) and color exchange responses (Fig. 3O). The high sensitivity to contrast and the sharp null near the isoluminant point (Fig. 3O) is indicative of neurons that have a dominant input from the magnoellular division of the lateral geniculate nucleus (Shapley and Hawken, 1999). Fig. 3P shows records of the extracellular spike waveforms. As mentioned in Section 2, we recorded the continuous filtered signal using Spike2 (Cambridge Electronic Design) software. These records were acquired during the initial characterization phase (Fig. 3P left, upper panel), the labeling phase (Fig. 3P middle, upper panel) and the re-characterization phase (Fig. 3P right, upper panel) of the recording. This was done in order to monitor the health of the cell through the period that it was loose-patched and also to maintain a record of good isolation. This record also provides data for spike shape analysis and its correlation with the morphological and functional subtype of the cell (see, for example, Henze et al., 2000). Example waveforms from the spike trains shown in Fig. 3P (upper panel) are shown in Fig. 3P (lower panel) on an expanded timescale indicating that the spike shape is maintained during the three phases. We have also found that the extracellular spike shape can differ between neurons. Extensive analysis of spike shape suggests that inhibitory interneurons have faster initial rise and decay than regular spiking pyramidal neurons (Henze et al., 2000). In Fig. 4A we show a comparison of the L5 pyramidal neuron spike waveform with that of a spine free presumptive inhibitory interneuron also from layer 5 (functional characterization data not shown here). A micrograph of the presumptive inhibitory interneuron is shown in Fig. 4B. This neuron was recovered 16.5 h after labeling. Our method of functional characterization, morphological identification and spike waveform comparison may offer a further means of separating neurons into functional subpopulations that make up local cortical circuits.

3.2.3. Layer 4α spiny stellate neuron—morphology

As an example of the quality of labeling attainable by this method, we show the micrograph and computer reconstruction of a spiny stellate neuron from layer 4Cα in Fig. 5A–D. This neuron was recovered 17 h after labeling. The laminar location of this neuron was ascertained by staining a section adjacent to that containing the soma for cytochrome oxidase (CO). In Fig. 5A, we show a reduced opacity photomicrograph of this neuron superimposed on a micrograph of the CO stained section. The location of this neuron in the upper half of 4C is evident. We traced the processes through thirteen 60 μm thick sections to obtain the dendritic and axonal distribution shown in Fig. 5B. The dendritic tree is symmetric and extends about 100 μm radially from the soma. A closer view of the dendritic arbor in the horizontal plane is shown in Fig. 5D. Here, the tracing was rotated so as to view the tree from above (i.e. in the horizon-

Fig. 4. (A) Comparison of the spike waveform from the L5 pyramidal neuron shown in Fig. 3 with that obtained from a neuron (B) with smooth dendrites and a beaded axon, a presumptive inhibitory interneuron from the same layer. Note the fast time course of the initial phase of the waveform of the smooth neuron. The smooth neuron was labeled with BDA.
Fig. 5. A BDA labeled 4Cα spiny stellate neuron. (A) A low-magnification micrograph showing the position of the labeled neuron in layer 4Cα. (B) Computer reconstruction of this neuron in the parasagittal plane; dendrite is shown in heavy lines. The axonal arbor has four terminal zones, all in layer 4B (shown by heavy arrows). Two of these terminal zones were found in layer 4B in sections distant from that containing the labeled soma of this neuron. In the two-dimensional projection shown here, these arbor (A2 and A3) are thus out of the plane of the dendritic arbor; the layer 4B boundary is indicated by the red arrows. (C) High power micrograph of the cell body and proximal dendrite and axon. Sp: spines; Ax: axon. (D) Tracing of dendritic tree rotated 90° about the Y-axis to give a view as seen from above looking down on the opercular surface of V1 (i.e. a view in the horizontal plane). This shows that the dendritic spread is approximately isometric. (E–J) These panels show the characterization of the functional properties of this neuron: (E) orientation tuning; (F) spatial frequency tuning; (G) temporal frequency tuning; (H) color exchange; (I) contrast response function; (J) area tuning.

tal plane). The symmetric spread of the dendrite about the soma is evident here. This neuron has major axonal projections to layer 4B and a projection to layers 5 and 6. There is a major terminal cluster in layer 4B directly above the cell body. There are three further terminal clusters (Fig. 5B: A1–A3) located about 700 μm from the soma. Due to the curvature of the cortical sheet, the layer boundaries observed in a single parasagittal plane may not correlate exactly with those from a section a few hundred microns away. The boundaries between layers 4A and 4B and between 4B and 4Cα were traced for each of the 13 sections, and segments of labeled axon or terminal clusters were located with respect to these local boundaries. Thus, while it is not visually evident in the 2D parasagittal projection shown in Fig. 5B, each terminal cluster was in fact located in layer 4B and we have indicated the local laminar boundary by arrows in Fig. 5B. The red arrows indicate that these axonal projections are not in the plane of the paper. Such a pattern of terminal clusters is consistent with the “lattice connections” described previously by Rockland and Lund (1983). Axon branches and their terminals from a single neuron in layer 4Cα, such as this example, may underlie such a lattice in layer 4B. A high power micrograph (Fig. 5C) shows the initial axon segment and a secondary axonal branch as well as profusely spiny proximal dendrites.

3.2.4. Layer 4Cα spiny stellate neuron—functional characterization

The tuning curves for this cell obtained before the labeling protocol was run are shown in Fig. 5E–J (solid lines). This neuron modulated its response to the drifting grating stimulus and had a modulation ratio (first harmonic divided by mean) of 1.3;
its receptive field would thus be classified as “simple” (Skottun et al., 1991) and we present the tuning in terms of this modulation (or first harmonic of the response; f1 amplitude). This cell responded at all orientations (Fig. 5E) with some preference for orientations in the range 160°–280°. The spatial frequency tuning (Fig. 5F) was low-pass but had a peak near 0.4 cycles/°. The temporal frequency tuning (Fig. 5G) was broad with a peak near 10 Hz. The high sensitivity to contrast (Fig. 5I) and a sharp null near the isoluminant point (Fig. 5H) is indicative of neurons that have a dominant input from the magnocellular division of the lateral geniculate nucleus (LGN; Shapley and Hawken, 1999). Layer 4Cox is known be the dominant recipient of input from the magnocellular division of the LGN (a smaller input is received by layer 6; Hendrickson et al., 1978). The functional properties of this neuron—high contrast sensitivity and a null near isoluminance are characteristic features of the magnocellular pathway. An interesting property of this neuron is seen in the size tuning function (Fig. 5J)—the response of the neuron is completely suppressed at the largest stimulus size. Tuning curves were obtained after labeling for the spatial frequency, temporal frequency and color exchange experiments (Fig. 5F–H; solid lines). These match well with those obtained before tuning. As we obtain the tuning, our standard procedure is to tailor the stimulus parameters, obtained from the preceding tuning curves, to elicit the maximal response from the recorded cell. Often this results in small shifts in the tuning curves obtained before and after the stimulus was optimized and this is seen in a few data points in the spatial frequency tuning curves.

3.2.5. Layer 4B spiny star pyramidal neuron—morphology

As a final example from our data, we present the computer reconstruction of a characterized and labeled layer 4B star-pyramidal neuron (Fig. 6). This neuron was recovered 24 h after labeling. The dendritic field (Fig. 6, heavy lines) is radial about the soma with the exception of a major apical dendrite extending into layer 2/3. The axon divides below the soma into four major branches. Two of these branches extend laterally in layer 4B, one extending at least 1.5 mm from the soma. A third branch projects to layer 2/3 while the fourth branch projects to layer 6 giving rise to long lateral branches in layer 5 and one branch in layer 4CB.

3.2.6. Sampling

We have used the LP–JC method to record from and label neurons in layers 2/3, 4A, 4B, 4Cox, 5 and 6. These neurons range from small cells of layers 2/3 (spiny pyramidal, aspiny non-pyramidal) and 4B (spiny stellate), through medium sized cells of layer 6 (pyramidal) to large cells of layers 4B (star pyramidal) and 5 (spiny pyramidal and aspiny non-pyramidal). Although we do not yet have a large sample, we have not found that we are preferentially obtaining characterized neurons in particular layers or based on size, except that we do not have cells from layer 4CB. Our method and others may be biased against obtaining layer 4CB neurons since the somata of the stellate neurons in this layer are less than 10 μm in diameter. Anderson et al. (1993) reported difficulty in recording intracellularly in vivo from the neurons in layer 4CB. Similarly, in describing their in vitro labeling experiments, Callaway and Wiser (1996) reported that neurons in 4CB proved to be the most difficult to fill intracellularly. Furthermore, in our experience with standard extracellular recording, we have found that it is difficult to obtain well-isolated single neurons in layer 4CB.

4. Discussion

We have described a method that allows us to record from and label single neurons in vivo in macaque primary visual cortex. The labeled neurons show detailed morphological features including the dendritic arbor, dendritic spines and axonal branching—both within cortex and into the white matter. The neurons show a range of visual tuning characteristics. We typically obtain stable recordings of 1–3 h from single neurons, which is sufficient to carry out a quantitative functional characterization and label the cell. We have recovered characterized and labeled neurons over 2.7 days after initial characterization and labeling. Thus, such a method is applicable in the acute macaque preparation, in which experiments can extend to several days. Recording at a range of depths through V1 cortex should allow for a sampling of neurons in the different layers and functional subpopulations. Links can then be made with existing knowledge about the anatomical organization of V1 (in terms of input and output layers), the various morphological classes of neurons found therein, their functional connectivity and visual response properties.

4.1. Comparison with other methods

All methods of recording and labeling have some distinct advantages and limitations. Here, we concentrate on the advantages and limitations as applied to the primate visual pathway,
particularly visual cortex. The preferred method would be intracellular recording throughout the experiment. This has not, as far as we know, been routinely applied to primate cortex. Sharp electrode experiments do not yield periods of stable recording that are sufficient to obtain comprehensive, quantitative functional characterization (Anderson et al., 1993). And attempts to make whole-cell recordings from primate V1 have proved exceedingly difficult (Henrie and Borg-Graham, personal communication) using the same techniques that are successful in cat cortex (Borg-Graham et al., 1996; Hirsch et al., 1995, 1998, 2002, 2003). Even in experiments using the technique pioneered by Martin and Whitteridge (1984a), extracellular recording with a sharp electrode to get the receptive field properties has not been used for quantitative assessment of a range of visual properties although the subsequent intracellular labeling does give excellent dendritic and axonal fills (Anderson et al., 1993).

In the juxtacellular method described by Pinault (1996) a glass sharp electrode (tip resistance 25–70 MΩ) is brought into close apposition to a neuron. The close proximity is judged by the ability of a train of current pulses applied via the electrode to modulate the neurons’ response to electrical stimulation of either its axon or of neurons presynaptic to it, via remote electrodes. With the electrode in close apposition to the neuron, labeling is obtained by passing current pulses of about 200 ms duration for 10–20 min. The current pulses are thought to electropropate the cell membrane and allow small protein molecules to enter the neuron. The electrodes are filled with conventional tract tracing molecules (neurobiotin) and the application of current pulses over a period of minutes results in single cell labeling. The process labels the soma, dendrites and axons of a single neuron. The depth of information that such methods can yield was demonstrated recently by use of Pinault’s technique in the rat hippocampus by Klausberger et al. (2003, 2005). They obtained complete fills with biocytin in vivo. In subsequent processing they used immunocytochemical labeling of the filled cells for calcium binding proteins and metabotropic glutamate receptors, resulting in identification of distinct classes of inhibitory neurons. The method we described here builds on the one developed by Pinault and is an extracellular version of the technique recently described for in vivo whole-cell recordings (Margrie et al., 2002). There are a number of details in our LP–JC technique that make it useful particularly in the primate. First, because we search for neurons using a ‘blind’ search method similar to that described by Margrie et al. (2002), we do not require the neuron to spike before we are able to make a loose-patch. Since we do not depend on being able to drive neurons before isolating them, we suggest that the LP–JC method is less biased than standard extracellular recording techniques towards recording from only those cells that can be mapped and driven with relatively simple stimuli (as is done in conventional experiments when searching for cells). Second, in our experience in primate V1, sharp electrodes, as used in conventional juxtacellular recording (Pinault, 1996), do not give stable juxtacellular recording of well-isolated spikes. The sharp electrodes either penetrate the cell if they are close enough to obtain a well isolated extracellular spike or the extracellular spike is small if the electrode is distant from the cell and therefore, with such electrodes, isolation becomes an issue.

We did not recover any of the neurons that we attempted to label with the higher currents (1–2 nA), after characterization, that have been reported to work for juxtacellular labeling (1–5 nA, Pinault, 1996). Instead, we have found that using low currents (0.3–0.5 nA) results consistently in successful labeling without causing a large increase in spontaneous activity or killing the cell. As discussed in Section 3, we often observe a small increase in the spontaneous activity (Fig. 31) although this returns to pre-labeling levels within 15–20 min. The layer 4Cα stellate neuron and the layer 4B star pyramidal neuron shown here were both labeled using a 0.5 nA current. As can be seen from the micrographs (Figs. 3B–D, 4B and 5A, C) and reconstructions (Figs. 3E–H, 5B, D and 6), this current amplitude is sufficient to produce extensive labeling of the axon and dendrites.

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