Intracellular and Juxtacellular Staining with Biocytin

Intracellular and juxtacellular methods for staining neurons allow microscopic visualization of neurons during electrophysiological experiments. These methods label all processes of a neuron, including the cell body, axon, dendrites, and dendritic spines. When sufficient time is allowed for transport of biocytin into the cell, the axon may be followed for several millimeters, permitting complete visualization of axonal arborizations in some cell types. The approach described in this unit is an indirect method in which biocytin or a related molecule is injected into the cell and is later revealed via high affinity avidin-biotin binding. This is in contrast to vital staining in which a visible substance (such as Lucifer Yellow) is injected directly into the cell. The biocytin method has been widely used since its introduction in 1988 by Horikawa and Armstrong. It is a highly sensitive, very reliable, and versatile method. Biocytin is not visible under the microscope, therefore, the method requires subsequent staining using simple histochemical methods. The intracellular staining protocol can be performed in vivo or in slices (Horikawa and Armstrong, 1988; Kawaguchi et al., 1989; Kawaguchi, 1992, 1993) and consists of obtaining stable intracellular recordings from a neuron and injecting tracer into the neuron. With juxtacellular staining in vivo, the electrode tip is apposed to the membrane of a neuron and tracer is applied extracellularly (Pinault, 1994, 1996; Zheng and Wilson, 2002).

These methods make identification of individual neurons using microelectrodes routine, without compromising the quality or duration of the primary physiological experiment. Because there are so many different ways of obtaining microelectrode recordings, this unit does not cover the details of the physiological method, but begins from the point of having a suitable physiological preparation in which such recordings are possible. Three main kinds of recordings are described: (1) sharp electrode intracellular recording (see Basic Protocol 1), (2) whole-cell intracellular recording (see Alternate Protocol), and (3) juxtacellular (extracellular) recording (see Basic Protocol 2). All of these recordings could be performed almost identically in vivo or using slices (or explants). The method of preparing tissue (see Support Protocol) for histochemical processing is different for these preparations, but the histochemical steps for staining neurons after preparing the tissue are identical.

CAUTION: Osmium tetroxide is a hazardous chemical; wear protective gear, use in hood, avoid spilling, and store in an airtight container. Also, use gluteraldehyde, picric acid, and paraformaldehyde in the hood.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

INTRACELLULAR BIOCYTIN STAINING WITH SHARP ELECTRODES

Intracellular recording with sharp electrodes is technically demanding and investigators are often unwilling to compromise on the procedures required to obtain secure recordings for the sake of intracellular staining. However, both recording and the intracellular staining technique described here have the same requirement of stable, long-term recordings, therefore, there is no need to make procedural compromises.
Biocytin or Neurobiotin

A solution of 1% to 4% (w/v) biocytin (372.5 mol. wt., Sigma) or Neurobiotin (322.8 mol. wt., Vector Laboratories) in 0.5 to 3 M potassium acetate, potassium chloride, or potassium methylsulfate should be prepared. Low salt concentrations (0.5 to 1.0 M) are recommended for more effective transfer of biocytin into cells, and to reduce alteration of the potassium equilibrium potential of the cell under study. Higher salt concentrations yield lower electrode resistances and may be required when using very fine-tipped sharp electrodes. Heat solutions (to ~50°C) to facilitate dissolution of biocytin or Neurobiotin. Biocytin is used by more investigators than is Neurobiotin, but Neurobiotin is more soluble and may be more effectively ejected by iontophoresis. Both forms work well for intracellular staining. A typical solution in a microelectrode is 1 M KCl and 2% biocytin or 2% Neurobiotin.

Micropipets and Filling

Glass micropipets should be selected to obtain optimal intracellular recordings for the physiological experiment. Filling micropipets with solutions containing biocytin or Neurobiotin is slightly more difficult than filling with simple salt solutions primarily because the biocytin-containing salt solution is slightly more viscous so air bubbles are more likely to become trapped in the electrode. Gentle tapping on the outer wall of microelectrodes dislodges most trapped bubbles. If the micropipets are pulled from filament-containing glass, this will probably not be noticeable. Electrodes can be filled from the shaft, replacing the usual pipet solution with one containing biocytin (or Neurobiotin).

Figure 1.12.1  Biocytin-filled cell. This is a pyramidal cell recorded in layer V of primary somatosensory cortex with electrodes filled with 3% biocytin and 1 M potassium acetate. The cell was stable for 1.5 hr, and was stained for 40 min with 1-nA current pulses (100 msec) delivered at 3 Hz. The axon of this neuron was followed out of cortex through the striatum to a terminal site in the perirhinal cortex. Scale bar = 50 μm.
**Biocytin Ejection**

Biocytin can be ejected by application of either positive or negative currents using a constant current device such as that provided with an active bridge intracellular recording amplifier (e.g., from Cygnus technologies; also known as a Neurodata amplifier, Ir183 or 283). Recommended currents range from 0.25 to 5 nA, with a duty cycle of 50% (e.g., 300 msec on and 300 msec off), for 15 to 90 min. The ejection current should be adjusted so that it does not damage the cell. The most reliable predictor of good staining is stable and secure intracellular recording throughout the biocytin ejection period (Figure 1.12.1). It is important to terminate ejection current if there is a substantial deterioration of the intracellular recording. Continuing to eject biocytin into an obviously damaged cell (this is sometimes called kill-and-fill) is likely to result in staining of multiple neurons. After filling a cell, the survival period can be anywhere between 1 and 24 hr. Survival times >24 hr are counterproductive due to intracellular degradation of biocytin.

**INTRACELLULAR BIOCYTIN STAINING WITH PATCH ELECTRODES**

Intracellular recording with patch electrodes requires no special procedure beyond adding biocytin or Neurobiotin to the patch solution, and the exercise of caution in the application of positive pressure to the micropipet. The most likely serious problem is damage to the recorded cell that can lead to its degeneration.

**Biocytin or Neurobiotin**

Add 0.1% to 0.5% (w/v) biocytin (Sigma) or Neurobiotin (Vector Laboratories) to the usual patch solution (see Basic Protocol 1).

**Micropipets and Filling**

To prevent inadvertent staining of the surrounding tissue, it is useful to fill the tip with biocytin-free patch solution by application of negative pressure to the electrode while immersing the tip in biocytin-free solution. Fill the electrode shaft with biocytin-containing solution, and remove bubbles.

**Biocytin Ejection**

No ejection is required, as the biocytin exchanges into the cell by diffusion.

**JUXTACELLULAR BIOCYTIN STAINING**

Juxtacellular staining is initiated from an extracellular position, and so employs the micropipets and filling solutions of extracellular recording (Pinault, 1996). The most difficult step in the juxtacellular method is recognizing when the electrode is in the correct position relative to the recorded neuron. Extracellular recording is not always done using glass micropipets or active bridge amplifiers. For juxtacellular staining, glass micropipets are required and the use of an active bridge amplifier is strongly recommended.

**Biocytin or Neurobiotin**

Prepare a solution of 1% to 4% (w/v) biocytin or Neurobiotin in 1 M NaCl.

**Micropipets and Filling**

The sizes and shapes of micropipets used in extracellular recording experiments are not as critical as those for intracellular recording. Pipets should be constructed in a way consistent with obtaining good extracellular recordings of the neurons under study. There should be a good signal-to-noise ratio and the electrode characteristics should make it easy to distinguish between action potentials recorded from two adjacent neurons. It is common to use pipets with diameters of 1 to 5 µm. Filling such electrodes is not difficult, although construction of pipets from filament-containing glass capillaries is recommended. There are no special precautions to prevent leakage of biocytin from the tip.
Because biocytin can be ejected by either negative or positive current, application of a holding current while searching for cells is not recommended.

**Biocytin Ejection**

Juxtacellular staining requires movement of the electrode into a position that facilitates staining. The ideal position of the micropipet for staining the cell is not the common configuration for extracellular recording. For stable extracellular recordings, the electrode is usually backed as far away from the cell as possible while maintaining a good signal-to-noise ratio. This reduces the likelihood of damaging the cell with the micropipet. For juxtacellular labeling, the micropipet must be positioned as closely to the cell as possible. To achieve the correct position, the electrode is advanced until the extracellular action potential amplitude is at its maximum. At this point, the active bridge amplifier is used to pass positive (anodal) current pulses, with a 200- to 500-msec duration, 50% duty cycle (e.g., 300 msec on 300 msec off), starting at an amplitude of 1 nA. The amplitude of this current is gradually increased (should not exceed ∼10 nA) while the neuron is monitored for changes in electrical activity. The electrical current pulses induce the juxtacellular configuration of the electrode, indicated by a sudden increase in background noise. The microscopic details of this phenomenon are not known, but it is probably comparable to a loose on-cell patch configuration. In some neurons, this is accompanied by a sudden increase in sensitivity of the cell to the current pulses. That is, the cell may begin to fire in response to the current pulse. Action potentials at this point will be of large amplitude (1 to 10 mV). Regardless of whether the cell begins to fire, the current amplitude should be quickly reduced when the change in background noise is observed, otherwise the cell is likely to be irreversibly damaged. Ejection currents in the range of 1 to 5 nA can then be continued for 15 to 90 min to stain the neuron while monitoring the background noise and cell firing to insure that the micropipet has remained in the juxtacellular configuration. Occasionally, when the juxtacellular configuration is lost during the injection, it can be recovered by momentarily increasing the current amplitude as before.

Not every attempt to achieve the juxtacellular configuration is successful. Simply ejecting biocytin into the extracellular space almost never stains a single neuron. Either several cells and processes are stained or no processes are stained.

**TISSUE PROCESSING**

Tissue handling varies somewhat between in vivo and slice preparations. For slices, it is recommended that the brain be perfused (UNIT 1.1) with slice medium before slices are prepared to reduce background staining. This is most important for the DAB method for visualizing biocytin because of the peroxidase activity of hemoglobin. In vivo, perfusion fixation is preferred for the same reason. Slices should be carefully removed from the recording chamber and fixative poured over them carefully as they lay flat on the bottom of a glass vial. The following protocol assumes fixation in the most generic fixative, 4% formaldehyde (see UNIT 1.1), but the biocytin method is compatible with a variety of fixatives so the recipe can be varied widely to meet requirements of the experiment. It is important to use a cross-linking fixative to immobilize the biocytin and prevent it from washing out of the cell during tissue processing.

Biocytin diffuses readily within cells. The soma, dendrites, and local axonal branches of most cells will be stained at all practical survival times (from 30 min up to 20 hr after staining). If staining is done to visualize these parts of the neuron, there is no reason to extend the survival time beyond the time required for ejection of biocytin. If long axonal branches must be stained, increasing survival time to 12 to 16 hr can be desirable. There
is little value in going much beyond 12 to 16 hr, as staining intensity is compromised by clearance of biocytin from the cytoplasm.

**Materials**

- **Tissues**
  - 0.1% Triton-X 100
  - Phosphate-buffered saline (PBS), pH 7.2
  - ABC kit or avidin-conjugated fluorescent marker (e.g., avidin-Texas Red)
  - 0.05% (v/v) 3′,3′-diaminobenzidine (DAB)
  - 3% (v/v) hydrogen peroxide
- Vibratome or freezing microtome
- Shaker
- Additional reagents and equipment for dehydrating and mounting sections (*UNIT 1.1*)

**Section and permeabilize tissue**

1. To facilitate penetration of labeled avidin into the tissue containing the biocytin-labeled cell, section tissue on a vibratome to a 50-µm thickness so that it makes it likely that the cell is near one surface.

   *Detection of biocytin is based on its high affinity for avidin, which is a large molecule that does not readily penetrate fixed tissue.*

   *Vibratome-cut sections are preferred due to their superior preservation of cell structure, but sections may be cut in a variety of ways. Sections should be kept in serial order. Tissue slices used in slice experiments may be stained as whole mounts or re-sectioned using a vibratome or freezing microtome.*

2. Permeabilize the tissue sections by incubating in 0.1% Triton-X 100 in PBS for 1 hr on a shaker at room temperature.

   *Thicker sections have the advantage of containing larger fractions of the stained neuron and its processes. Sections up to 250-µm thick can be stained successfully by increasing the permeabilizing treatment by increasing the Triton-X 100 concentration to 1% and increasing the incubation time to 8 to 12 hr.*

**Stain sections**

3. Incubate in ABC solution (1:100 in PBS containing 0.1% to 0.2% Triton-X 100 mixed according to kit manufacturer’s instructions) or avidin-Texas Red or other avidin-conjugated fluorophore (1:100 in PBS containing 0.1% Triton-X 100). Incubate overnight at room temperature for sections that are 150- to 200-µm thick; otherwise incubate 2 to 4 hr at room temperature.

4. Wash several times in PBS, pH 7.2.

5. For sections treated with ABC solution, incubate in 0.05% 3′,3′-diaminobenzidine and 0.003% hydrogen peroxidase in PBS ~20 min, monitoring staining by microscopic examination of wet sections. If additional time in the DAB solution is required, use repeated 20-min incubations, using freshly mixed solution each time.

6. Wash repeatedly in PBS.

7. For sections treated with avidin-Texas Red or other fluorescent avidin conjugate, it is possible to view immediately. If desired, subsequently treat these sections in ABC and react with DAB to make more permanent preparations (see step 5).

8. Dehydrate and mount (*UNIT 1.1*), or embed for electron microscopy.
Intracellular and Juxtacellular Staining with Biocytin

1.12.6

COMMENTARY

Background Information

The interpretation of neurophysiological recordings relies on some knowledge of the neuron under study. A number of methods are employed to identify cells. Antidromic activation of neurons by stimulating a known target structure is a classic method for identifying cells. Recently, it has become possible to identify neurons in the course of the slice experiment by the appearance of their somata and proximal dendrites during differential interference contrast (DIC) microscopy. Occasionally, previously established physiological features of particular cell types can be used, but this assumes that a previous study allowed a more perfect identification. The definitive identification of cell types in the nervous system is morphological. Somatodendritic morphology or an axonal projection pattern can establish the identity of a neuron. In exceptional cases, cytochemical markers are necessary to distinguish cell types. Using intracellular staining, cell identification by physiological criteria, morphologic reconstruction, and cytochemical identification can all be brought to bear on single neurons recorded in vivo or in slices (e.g., Kawaguchi, 1993).

Sharp electrodes

The sharp electrode approach to intracellular recording relies on impaling neurons with fine-tipped micropipets that have small apertures, and minimal exchange of solutions between the cell cytoplasm and the microelectrode. The assumption that there is no solution exchange is used to justify the use of nonphysiological solutions (e.g., 3 M KCl) in the micropipets. This assumption is often not justified, and cell properties can be altered by ions present in the micropipets (e.g., Nisenbaum and Wilson, 1995), but biocytin present at practical concentrations in sharp electrodes (1% to 4%) cannot be expected to reliably diffuse into the cell in quantities required for good staining.

The percentage of biocytin and Neurobiotin is not as critical to staining a neuron well as is the duration of staining. The choice of potassium counterion (i.e., acetate, chloride or methylsulfate) will depend on the experiment (e.g., using KCl to alter the reversal potential of IPSPs) and will have little bearing on how well a neuron is stained. To ensure good staining, it is necessary to facilitate movement of biocytin by injecting current through the micropipet. At neutral pH, biocytin will move from the electrode with either polarity of current. In intracellular recording, currents passed through the micropipet must traverse the cell membrane as well, so the choice of current polarity and intensity must take into account the peculiar sensitivity of individual cell types. The spinal motoneuron, for example, with a rheobase of several nanoamperes offers the investigator impressive latitude in the choice of biocytin ejection protocols. Other neurons have their own sensitivities. Many neurons, like the striatal spiny projection cell, can tolerate being depolarized to the point of high frequency firing for long periods of time but will rapidly deteriorate if artificially hyperpolarized. Many cells more readily tolerate current ejection if it is not continuous. A common procedure is the use of current pulses, usually 100 to 500 msec long, with equal periods in which no current is injected. Although positive- or negative-going currents can be used, the most reliable results are obtained by consistent use of one or the other, rather than alternating positive and negative pulses. There is a trade-off between current magnitude and ejection time. For cells that do not tolerate strong currents, one can make up for it by injecting a current for longer periods of time.

A dangerous side effect of current injection as part of an intracellular recording protocol is the inadvertent staining of additional neurons. Usually, this is not a problem because every cell from which data is to be collected must be stained. However, some investigators are in the habit of passing current (usually hyperpolarizing) during the initial period after impaling a cell to prevent action potentials as the recording stabilizes without realizing that this action can also lead to cell staining. Whether intended or not, this procedure can lead to a stained neuron, but it is not a reliable way to inject neurons. It is recommended such practices be avoided when using biocytin-containing electrodes. Passing constant current to quiet a neuron is more of a tonic for the nerves of the investigator than it is for the neuron under study.

The choice of electrodes in sharp recording must be dictated by the requirements for stable recordings. Usually, investigators do not wish to alter the configuration of the micropipets to facilitate intracellular staining. Biocytin is a small (372.5 mol. wt.) molecule that can be effectively ejected from even the smallest aperture pipet tips usable for microelectrode recording. Its utility as an intracellular stain was
realized by Horikawa and Armstrong (1988) in their search for a replacement for horseradish peroxidase (≈40 kD mol. wt.), which was not performing satisfactorily with the fine-tipped micropipets necessary for use with supraoptic neurons in hypothalamic explants. Increasing the concentration of biocytin in the pipet solution (up to a maximum of ≈4%) increases the likelihood of staining a neuron. Reducing the salt concentration can have the same effect.

**Patch electrodes**

The patch electrode tip is usually much larger than the sharp electrode but this does not pose any special problems. The electrode and the cell exchange internal solutions, including biocytin.

**Juxtacellular staining**

The nature of the juxtacellular configuration is not well understood. The large-amplitude extracellular action potentials and background noise are consistent with a close approach of the micropipet aperture to the cell membrane (sometimes called a loose patch). Staining is probably due to local and reversible disruption of the cell membrane under the influence of the current (electroporation). The role of current pulses in achieving the juxtacellular configuration is also not known. The mystery surrounding key features of this method and the poor understanding of the underlying principles raises some concerns about the reliability of the method.

If cellular identification is critical and intracellular recording is possible, it is advisable to go the extra step and use intracellular recording and staining. However, there are many cell types in the brain that are relatively easy to study extracellularly in vivo, but are technically difficult to impale and hold with an intracellular electrode. An example is the projection neuron of the globus pallidus. These neurons are almost always immediately killed upon impalement by a sharp microelectrode. Juxtacellular staining of these neurons has revealed much about their morphology and axonal connections (Bevan et al., 1998).

**Tissue processing**

Biocytin, like biotin, forms a high affinity association with avidin, and this is the basis of its usefulness in staining. Fluorescent markers conjugated to avidin offer a direct way to visualize biocytin in very few steps. ABC (which is a complex of avidin, biotin, and horseradish peroxidase) offers a brightfield alternative employing enhanced sensitivity, more permanent preparations (which do not fade), and compatibility with electron microscopy (e.g., Xu et al., 1992). If fluorescent microscopy is preferred for light microscopy but subsequent electron microscopy is required, it is possible to use an avidin-conjugated fluorophore for light microscopy, followed by ABC incubation and DAB treatment for electron microscopy. This works because the ABC reagent is a complex of avidin, avidin, and horseradish peroxidase, which will bind to both avidin or biotin in the tissue. Avidin-conjugated fluorophore molecules bound to biocytin in the stained cell have free biotin binding sites, to which the ABC complex will bind.

There are several variants of the DAB reaction that employ metals to intensify or alter the color of the diaminobenzidine reaction product (e.g., Zheng and Wilson, 2002). These modifications can be useful for single neuron staining, especially if the tissue is to be counterstained using enzyme histochemistry or immunocytochemistry. Osmium treatment, used for electron microscopic preparations, also provides an effective intensification of the reaction product.

**Critical Parameters and Troubleshooting**

**Sharp electrodes**

The most common problems with staining include faintly stained (or unstained) cells, degradation of the stained neuron (due to damage), or staining of more than one neuron. If the histochemical procedure is working correctly (see below), faint but otherwise good staining is probably due to insufficient ejection of biocytin. In these cases, longer ejection times (30 to 90 min instead of 10 to 20 min) or higher currents (1.5 to 3.5 nA) are indicated. If more than one cell is stained, consider whether cells might be connected by gap junctions. As inviting as this interpretation may be, it is more likely that the electrode formed a poor seal with the cell and the biocytin leaked out of the injected cell into other neurons whose membranes were disrupted by the electrode. Multiple stained neurons were common when intracellular staining was done primarily with horseradish peroxidase, a large molecule that probably does not cross gap junctions. For the physiologist interested in studying a particular class of cells, staining more than one neuron at a recording site is a disaster. Which one, if any, is the cell from which the physiological data
were collected? If it is possible to stain a cell other than the desired one, what assurance is there that the recorded neuron is stained at all? It could have degenerated and disappeared.

The partly degenerated stained cell is another relatively common problem. Intracellular recording is a challenge to the life of the neuron. If the experiment damages the cell, it is common to see a crenelated or oddly shaped soma with large indentations, and swollen, discontinuous, or varicose dendrites. This can happen very rapidly; reducing the survival time is likely to have little effect. Axons are much more robust and take much longer to show signs of damage. It is possible for the soma and dendrites to be completely destroyed and absorbed, leaving a completely normal-looking axon of the intracellularly stained neuron, and leaving a dusting of lysosomes in the nearby neurons and glia. This kind of damage can usually be avoided by paying more attention to the condition of the neuron during the biocytin injection period. Unlike other intracellular markers (including horseradish peroxidase), biocytin itself does not seem toxic. Very intensely stained neurons can survive for days after injection and exhibit normal morphology.

**Patch electrodes**

The most likely problem is that patch solution sprayed from the electrode upon application of positive pressure while approaching a cell will contain biocytin, thus producing a strong, diffuse biocytin staining of the tissue along the path of the electrode. Biocytin can also be taken up by neurons or glia, staining cells other than the one intended. This is of special concern for blind recording in slices or in vivo. In blind recording, there may be a substantial search period in which the electrode is under positive pressure while in the tissue. In visualized recording, this period can often be shortened to only a few seconds and the amount of positive pressure can be minimized.

Another potential problem arises from damage to the cell by dialysis of its contents, or when pulling the electrode away from the cell to terminate the recording. In whole-cell recording it is common for cells to be seriously damaged simply by exchange of solutions with the electrode. Often the experiment is terminated by the demise of the cell due to dialysis. To insure good staining, this kind of exhaustive (for the cell) recording strategy must be curtailed. Shorter experiments or smaller electrodes and reduced dialysis (at the expense of increased access resistance) are indicated.

Damage done to the neuron when pulling the micropipet off the cell is also not usually a concern in whole-cell recording experiments. Because it can lead to rapid degeneration of the cell, traumatic termination of the whole-cell configuration should be avoided. This is usually accomplished by pulling off in a shearing direction (perpendicular to the direction of approach to the cell) or at least by pulling off gently. In experiments employing the on-cell configuration or in perforated patch recording, it is still possible to achieve good biocytin staining simply by breaking into the cell at the end of the experiment. Only a short time (5 to 10 min) is required to obtain good filling of the cell with biocytin.

**Juxtacellular staining**

The most difficult step in the technique is learning to recognize the juxtacellular configuration of the electrode. Different kinds of neurons respond to this technique differently, so no single, general set of criteria can be applied. For corticostriatal cells, for example, the absence of firing in response to the current pulses caused the authors much consternation (Zheng and Wilson, 2002). It appears that the change in background noise is a suitable criterion; the change in firing rate of the neuron is not necessary. The failure to achieve the correct electrode configuration is typically recognized after sectioning the brain and staining the sections. Two contrary outcomes both result from the failure to achieve correct juxtacellular configuration, (1) no stained cells are found; or (2) many neurons get stained where only one was expected. Presumably, the first occurs because biocytin was deposited extracellularly and was taken up or washed out of the tissue. The second could occur through filling of cell processes close to the electrode tip at the time of ejection. It should be remembered that, despite appearances during electrical recording, the neuropil is a crowded place with many cell processes surrounding the pipet tip at all times.

**Tissue processing**

Distortion of the tissue during tissue preparation is a common difficulty in tissue preparation. The most recalcitrant problem is shrinkage of the tissue. Not only does the tissue shrink but stained cells, at least those stained with DAB shrink less than the surrounding section. Differential shrinkage distorts cell processes, wrinkles them, or even breaks them. In the most extreme case, cell processes break at constant intervals, giving them a dotted line appearance.
Most shrinkage occurs during drying or dehydration of tissue sections. Sections that are dried onto subbed slides tend not to shrink along the plane of the slide because they are attached to the gelatin. Instead they collapse in the plane perpendicular to the slide, losing ~75% of their thickness during drying. If free floating during dehydration, shrinkage is more uniform, but can be just as destructive. One solution is to permeabilize all cell membranes before chemical dehydration or drying. Free-floating sections prepared for electron microscopy are treated with aqueous solutions of osmium tetroxide. This option is open to light microscopists as well, but much lower concentrations of osmium tetroxide should be used to prevent excessive darkening of the tissue. Tissues can be protected against shrinkage by treatment with osmium tetroxide at concentrations much lower than those usually used for electron microscopy. Treatment of 50-µm sections for 1 hr in 0.1% osmium tetroxide in phosphate-buffered saline, for example, provides good protection against shrinkage. Treatment with osmium tetroxide renders the tissue sections brittle; sections should be left flat during the osmium treatment and should be handled carefully afterwards. Osmium tetroxide penetrates tissue very slowly, so incubations should be long, (at least 1 hr) and concentrations should be kept low. A shorter treatment in a higher concentration will turn the section dark near the surface, and offer no protection against tissue distortion near the center.

**Anticipated Results**

**Sharp electrodes**

The cell body, dendrites, and dendritic spines of a neuron can typically be discerned by 10 to 20 min of staining, but longer staining is necessary to resolve the axon.

**Patch electrodes**

The cell body, dendrites, and dendritic spines of a neuron can be discerned without much effort. Because these slices are prepared before staining the cell, the dendrites and axons of these cells will typically be incomplete.

**Juxtacellular staining**

A single cell should be stained well by this procedure.

**Time Considerations**

**Sharp electrodes**

The most important consideration is the duration of current injection into the cell. Long durations (30 to 90 min) produce a darkly stained cell body and peripheral processes. The survival period post-staining can be <1 hr or up to 20 hr. Once the brain has been removed, sectioning should typically follow within 1 or 2 days. Then depending on the section thickness, the duration of treatment with Triton X-100 and the incubation in the avidin-biotin complex must be varied.

**Patch electrodes**

Once the slices have been placed in fixative, sectioning should typically follow within 1 or 2 days. If the 300-µm thick slice is to be stained without sectioning, then staining should follow 1 day after post-fixation. Depending on the section thickness, the duration of treatment with Triton X-100 and the incubation in the avidin-biotin complex must be varied. Longer staining is necessary for thicker sections. If the ABC complex does not penetrate, the cells will not stain, or will stain incompletely.

**Juxtacellular staining**

The animal is typically allowed to live overnight after juxtacellular recording. The remaining time considerations are very similar for sharp-electrode intracellular recording and the juxtacellular recordings.

**Tissue processing**

The treatment with Triton-X 100 can be increased from 0.5 to 1 hr for thicker sections. The incubation with ABC for a 50-µm thick section lasts 2 hr; for thicker sections, it can be overnight at room temperature. The reaction with diaminobenzidine and 0.003% hydrogen peroxidase can be for 20 min and can be repeated with fresh solution for another 20 min if the cells are not darkly stained.

**Literature Cited**


Intracellular and Juxtacellular Staining with Biocytin

**1.12.10**

Supplement 26

Contributed by Charles J. Wilson and R.N.S. Sachdev

The University of Texas at San Antonio

San Antonio, Texas