Computational Modeling Methods for Neuroscientists

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8 Reconstruction of Neuronal Morphology

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The goal of this chapter is to describe methods for obtaining high-quality, accurate 3-D reconstructions of neuronal morphology for use in constructing computational models (chapters 10 and 11). Because the accuracy of any model depends on the structural characteristics of the neuron as well as its biophysical properties, capturing the lengths and diameters of neuronal processes and the locations of synapses is critically important for developing realistic models. There are numerous sources of error that can be introduced during histological processing and tissue sectioning, as well as during the processes of neuronal imaging and digitizing, that can affect the quality of the model. This chapter summarizes the techniques currently in use for obtaining morphological parameters of neurons at both the cellular and ultrastructural levels and provides suggestions for reducing the errors introduced during the process. A description and evaluation of reconstruction techniques that employ both open-source and commercial software programs for obtaining 3-D reconstructions are included. There is now a growing number of accessible archives of 3-D reconstructions of neuronal morphology for use by the neuroscience and modeling communities.

8.1 Neuron Staining Techniques and Tissue Preparation for Light Microscopy

Whereas precise measurements of synapse distributions require the use of electron microscopy, accurate reconstructions of neuronal branches as well the relative distributions of dendritic spines and axonal boutons can be obtained using light microscopy (see Shepherd and Harris, 1998 for discussion). Any one of a number of different labeling methodologies can be used to visualize neuronal structure at the light microscope level. Each technique has its advantages and disadvantages and thus the choice of a labeling method will depend on several factors, including the goal of the experiment, the source of the tissue (such as vertebrate or invertebrate), and the region of the nervous system to be examined. Labels that can be injected into the cell are preferred for modeling studies because the electrode that is used for dye injection can also be used to obtain electrophysiological data on the same

neuron. Here we begin our discussion by focusing on those labels that are most commonly employed for intracellular injections, and then we consider other staining techniques that also provide excellent visualization of neuronal structure at the light microscope level.

Staining Neurons by Intracellular Injection

In selecting a label, an investigator must first consider whether a dense product or a fluorescent label is most suitable. Compounds that produce dense products are most often chosen for the analysis of dendritic trees and axons at the light microscope level (because they do not undergo photobleaching) and for studies that require combined light and electron microscope imaging. Early investigators used horseradish peroxidase (HRP) to label vertebrate neurons either in vivo or in vitro (Claiborne et al., 1986, 1990; Ishizuka et al., 1995) and cobalt chloride to label invertebrate neurons (Bacon and Altman, 1977, Levine and Murphey, 1980, S. E. Johnson and Murphey, 1985). Currently, biocytin is the most common intracellular label for both vertebrate and invertebrate neurons when a dense product is required (Horikawa and Armstrong, 1988; McDonald, 1992; Heinrich et al., 1998; Pyapali et al., 1998; X. J. Sun et al. 1998; Jaeger, 2001).

Fluorescent dyes are preferred for studies that require visualization of the neuron during the labeling procedure or that require the use of a laser-scanning microscope to analyze both neuronal branching patterns and the details of other morphological features such as dendritic spines or axonal boutons. One of the first available dyes for intracellular labeling was Lucifer Yellow (W. W. Stewart, 1978). Although it is still used for quantitative studies of neurons in fixed slices (Duan et al., 2002, 2003), it is less than ideal for labeling studies. It is inefficiently excited with an argon-ion laser and undergoes relatively rapid photobleaching. More recently, fluorescent hydrazide and hydroxylamine derivatives have been developed for intracellular staining (Alexa dyes; Invitrogen Molecular Probes); they are photostable, they can be fixed with aldehyde-based fixatives, and they are water soluble.

Staining Vertebrate Neurons with Biocytin

Biocytin can be used for intracellular labeling either in vivo or in vitro (Pyapali et al., 1998; Chitwood et al., 1999; Staiger et al., 2004; for a detailed review, see Jaeger, 2001). Here we focus on the use of biocytin in brain slices.

To prepare tissue slices for in vitro work, the animal is deeply anesthetized and the brain removed. The region of interest can be quickly dissected away from the rest of the tissue or the brain can be left intact. If preferred, the anesthetized animal can be perfused first through the heart with ice-cold saline to remove erythrocytes; blood cells contain endogenous peroxidase and will stain with the procedures required to visualize the biocytin (or horseradish peroxidase; Claiborne et al., 1986). To optimize

viability, the tissue should be sectioned immediately; a vibratome is most commonly used. Slice thickness will depend on the type of neuron to be labeled, but most investigators cut sections that are between 300 and 400 μm. If the slices are much thinner, one risks severing many dendrites on most cortical cells (see chapter 9), and if they are thicker, the viability of tissue toward the center of the slice is compromised, owing to hypoxia. To allow dendritic spines to stabilize, slices should be allowed to recover for 3 hr prior to beginning the experiment (Kirov et al., 2004)

Recordings and injections can be made using either sharp electrodes or patch electrodes. Sharp electrodes filled with 2–4% biocytin (Sigma) or Neurobiotin (Vector Laboratories) by weight, dissolved in 1 M potassium acetate, have good recording characteristics, and either compound can be injected using positive current pulses of 100–200 ms duration and an amplitude of 1–4 nA applied for 5–10 min at 1–4 Hz after electrophysiological data are obtained (Pyapali et al., 1998; Jaeger, 2001). Slices should remain in the chamber for 15 to 60 min (depending on the size and complexity of the neuron) after the injection is complete to allow the label to diffuse throughout the dendritic tree. When patch pipettes are used in the whole-cell configuration, concentrations of biocytin or Neurobiotin between 0.1 and 0.5% are normally employed and, in most cases, the neuron will fill by passive diffusion over the course of 30 to 60 min (Chitwood et al., 1999; Wang et al., 2002; Staiger et al., 2004; Golding et al., 2005).

To ensure the probability of staining neurons with complete dendritic trees, neurons with somata located near the center of the slice should be selected (Claiborne et al., 1990; Rihn and Claiborne, 1990; Mainen et al., 1996; Carnevale et al., 1997). This is often difficult to accomplish when patch recordings are done under visual control because only cell bodies relatively close to the surface are easily imaged with infrared differential interference microscopy. Thus investigators must be extra vigilant in noting severed dendrites during the reconstruction process, as discussed in more detail later and in chapter 9. Two other caveats are important. Slices should not be allowed to remain in the chamber for more than the minimal time required for label diffusion. If a neuron begins to die before it is fixed, swellings can occur so that a labeled process looks like a string of beads (see figure 6.1 in Jaeger, 2001). These artifacts in some adult neurons, however, should not be confused with the normal dendritic varicosities that often characterize interneurons or neurons from young animals (Jones et al., 2003). It is worth noting that chilling brain slices can also cause beading that disappears once the slices are warmed (Kirov et al., 2004). In addition, after recordings and intracellular injections are completed, the electrode should be removed carefully so that the injected neuron sustains minimal damage to the cell body and proximal dendrites.

After label injection, slices are fixed in 4% paraformaldehyde and 1.25% glutaraldehyde overnight, washed in buffer, and sectioned at 100 μm with a vibratome. Prior

to reaction with avidin coupled to horseradish peroxidase, slices can be made more permeable with a quick-freezing protocol (Jaeger, 2001) and incubated in 0.5% H_2O_2 for 30 min to inactive endogenous peroxidases. Slices are then incubated in avidin-horseradish peroxidase (Vectastain ABCkit, Vector Laboratories, Inc., Burlingham, CA) for either 3 hr at room temperature, or overnight at 4 °C, for best results. Next, slices are incubated in a solution of 1 ml of 0.05% diaminobenzidine (DAB) and 1 mM $NiCl_2$ in 0.1 M phosphate buffer for 1 hr on a shaker table. Three to four drops of 3% H_2O_2 in buffer are added to each vial and the slices incubated an additional hour. After washing in buffer, tissue can be mounted in an aqueous mounting medium (Golding et al., 2005) or cleared in ascending concentrations of glycerol and stored and mounted in 95% or 100% glycerol. The use of glycerol for dehydration and clearing minimizes tissue shrinkage and the artifacts that accompany dehydration with alcohol series. If needed, the HRP & DAB product can be intensified by rehydrating the slices in descending concentrations of glycerol, followed by a wash in 50 mM Tris buffer (pH = 7.4). Slices are then incubated in 2 ml of 0.1% cobalt chloride, 0.1% nickel ammonium sulfate, and 0.05% DAB for up to 45 min. The slices should be checked periodically, and when nonspecific tissue darkening begins to occur, washed in buffer and cleared and mounted in glycerol. If long-term storage of stained neurons is desired, an alternative method of hardening the tissue with osmium can be used (Staiger et al., 2004). This method produces minimal shrinkage of the tissue and less fading of the stained neurons.

Staining Invertebrate Neurons with Biocytin

The techniques for intracellular staining of neurons in invertebrates with biocytin are essentially the same as those described here for vertebrate preparations. Intracelluar injections are done in situ in semi-intact animals. Best results are obtained when the preparation is perfused with oxygenated saline and the tracheae (air supply) are intact. In most cases, the ganglion containing the stained neuron can be processed as a whole mount using protocols similar to those described earlier; however modifications of the procedure are required to achieve good penetration of the reactants into the tissue. Insect ganglia are covered in a thick glial sheath that requires softening with protease or incubation with detergents (Staudacher, 1998). Invertebrate neurons are often much larger in size than vertebrate neurons, requiring longer injection and diffusion times for optimal staining. Sometimes injection with biocytin results in light or incomplete staining of the extensive dendritic trees, precluding a complete anatomical reconstruction of the cell.

Staining Invertebrate Neurons with Cobalt Chloride

Injection of cobalt chloride is an alternative method used to stain neurons in invertebrate ganglia and results in a very dark stain and complete filling of all dendritic processes. Cobalt chloride can be injected intracellularly with pulses of positive current

(5 nA at 1 Hz) for a brief period of time (1–2 min). Cobalt ions diffuse very quickly through the cell, so long injection or diffusion times are not necessary to stain the cell in its entirety (Levine and Murphey, 1980). Cobalt is highly toxic to the cell, so the tissue should be processed quickly following staining to avoid artifacts such as blebbing and swelling. The ganglion (or brain) containing the stained cell is dissected from the animal and immersed in saline saturated with hydrogen sulfide for 15 min. The tissue is rinsed in buffer and fixed in Carnoy's fixative (60% ethanol (95%), 20% chloroform, and 10% glacial acetic acid) for an hour. The tissue can be stored in 70% ethanol for up to a week before further processing. The tissue is then rehydrated in a descending series of ethanol and incubated in a solution of gum acacia, hydroquinone, and citric acid for an hour at 50 °C, in the dark. Silver nitrate is then added to the solution and the tissue is incubated in the dark until the staining develops. The tissue is dehydrated in ethanol, cleared in methyl salicylate, and mounted in Canada balsam. This method stains cells completely with a black precipitate; however, the tissue shrinks by 50% or more during fixation and dehydration. These preparations can be stored mounted in Canada balsam for years with no fading of the stain. Detailed methods for this procedure can be found in Bacon and Altman (1977) or S. E. Johnson and Murphey (1985).

It should be noted that while cobalt chloride can be successfully injected into invertebrate neurons, it can also be applied extracellularly. For example, cricket mechanosensory afferents can be stained with these same methods by introducing cobalt chloride into the lumen of a cut hair and allowing the cobalt to diffuse into the sensory neuron in the intact animal overnight at 4 °C (S. E. Johnson and Murphey, 1985; Jacobs and Nevin, 1991).

In some cases, injection of Neurobiotin or biocytin into invertebrate neurons results in the staining of multiple cells, via diffusion through gap junctions of electrically coupled cells. Alexa dyes, with their higher molecular weights, can be used as a substitute since they are confined to the injected cell (Fan et al., 2005). The electrodes filled with Alexa have good recording characteristics, although not as good as those filled with biocytin alone.

Alternative Staining Methods for Obtaining Neuronal Morphologies in Vertebrate Tissues

There are four other types of techniques in common use that do not require intracellular dye injection: Golgi impregnation techniques (Desmond and Levy, 1982), dextram amine staining (Megias et al., 2001), lipophylic dye labeling (Rahimi et al., 2003; Garza-Meilandt et al., 2006), and fluorescent labeling through expression of endogenous green fluorescent protein (GFP) in transgenic mice (Feng et al., 2000; Mizrahi and Katz, 2003). These latter methods produce excellent structural data although they do not allow the measurement of physiological parameters.

The Golgi Method

Golgi impregnation in fixed tissue produces random staining of multiple neurons within the tissue. Although this method is less suitable for modeling studies, neurons are stained intensely and completely, albeit in an unpredictable fashion. This technique is useful for anatomical studies requiring tissue that is difficult to obtain, such as human pathology specimens. The high contrast between the stained neuron and surrounding tissue makes the anatomical reconstruction process easier than with other more lower-contrast methods (see K. M. Brown et al., 2005). For detailed protocols of the rapid Golgi technique, see Sultan and Bower (1998) or Desmond and Levy (1982).

Biotinylated Dextran Amine

Another useful label that results in a dense product is biotinylated dextran amine or BDA (Molecular Probes). BDA is injected into the brain region of interest and after a survival period the animal is anesthetized and perfused, the brain is sectioned, and the sections are processed for visualization of the stained neurons (for detailed methods, see Megías et al., 2001). This methodology results in Golgi-like labeling of single neurons, although often large numbers of neurons close to the injection site are labeled. BDA is particularly advantageous for those experiments requiring both light and electron microscopy.

Labeling with Lipophilic Dyes

Lipophilic dyes such as DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes) can be used to label cells in live or fixed tissue, producing an intensely fluorescent label that can be used with confocal imaging techniques and subsequent reconstruction from confocal images (described later). Most commonly, DiI labeling is done in fixed tissue. To prepare the tissue for labeling, animals are anesthetized and perfused with cold 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.4). The brains are removed and left in the cold fixative for 1 hr and then washed in buffer. The fixation time should be closely monitored because longer fixation times lead to less than optimal labeling. Brain slices (200–400 μm) are cut using a vibratome and the thickness of the slice should be chosen to ensure that an entire neuron is contained within the slice. Crystals of DiI are crushed and small crystals are picked up on the tip of a micropipette and placed directly on the slice in proximity to the neurons of interest. For example, to label pyramidal neurons in hippocampal region CA1, crystals are placed at the top of the stratum lacunosum-moleculare, the layer containing the distal dendritic tips (Garza-Meilandt et al., 2006). Labeled slices should be stored in 2% paraformaldehyde for 2–3 days to allow diffusion of the dye throughout the neurons and then mounted in buffer; because DiI is lipophilic, alcohol- and glycerol-based mounting media cannot be used. Labeled neurons can be imaged with a confocal microscope

and the neurons reconstructed from the image stacks as described later (Rahimi et al., 2003). It is worth noting that although DiI labeling is often most impressive in tissue from young animals (Jones et al., 2003), recent studies show that it can produce excellent labeling in tissue from young adult and aged rodents (Kirov and Harris, 1999; Rahimi et al., 2003; Garza-Meilandt et al., 2006).

Fluorescent Labeling with Green Fluorescent Protein in Transgenic Mice

Many lines of transgenic mice have been developed that express GFP in specific populations of neurons (Feng et al., 2000; Mizhra and Katz, 2003). Varying numbers of neurons express GFP in the different lines. Two lines are particularly useful for quantifying the morphology of hippocampal neurons: the "M" line and the "O" line (Feng et al., 2000). In these lines, GFP is expressed under the control of neuron-specific elements from the thy1 gene, an immunoglobulin gene that is expressed by projection neurons in many parts of the nervous system, as well as in some non-neuronal cells. Deletion of a specific intron abolishes expression in non-neuronal cells. Excellent GFP expression is found in 24-month-old mice (Perez et al., 2005).

To prepare the tissue, animals are deeply anesthetized with pentobarbital (130 mg/kg) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4). The brain is removed from the skull, left in the fixative for 3 hr, washed in buffer, and sectioned coronally using a vibratome at 200 μm. The sections are mounted in Vectashield (Vector Inc., Burlingame, CA). Fluorescently labeled neurons can be examined using a microscope equipped with a fluorescent attachment, the appropriate filter (DiI, Chroma No. 41002; GFP, No. 31001; Chroma Technology Corp., Brattleboro, VT), and a 63× Zeiss oil immersion objective (NA 1.25; 0.5-mm working distance). After applying selection criteria, acceptable neurons are imaged with a confocal laser scanning microscope and dendritic trees reconstructed as described later (figure 8.1).

Alternative Staining Methods for Obtaining Neuronal Morphologies in Invertebrate Tissues

GFP has been used extensively for studying neuronal morphology in invertebrates, most commonly in *Caenorhabclitis elegans* (Chalfie et al., 1994) and in *Drosophila* (Brand, 1995) either as an endogenously expressed protein or in conjunction with the GAL4 system, which permits in vivo labeling of neurons and other cell types throughout development (Brand and Dormand, 1995; Ito et al. 1997). GFP can also be used to label the entire structure of neurons homozygous for specific genes in the intact brain using the mosaic analysis with repressible cell marker (MARCM) technique (Lee and Luo, 1999). Neurons labeled in this way can be reconstructed from stacks of confocal images for morphometric analysis. In experiments in which it is important to view the GFP-labeled neuron in conjunction with the ganglion

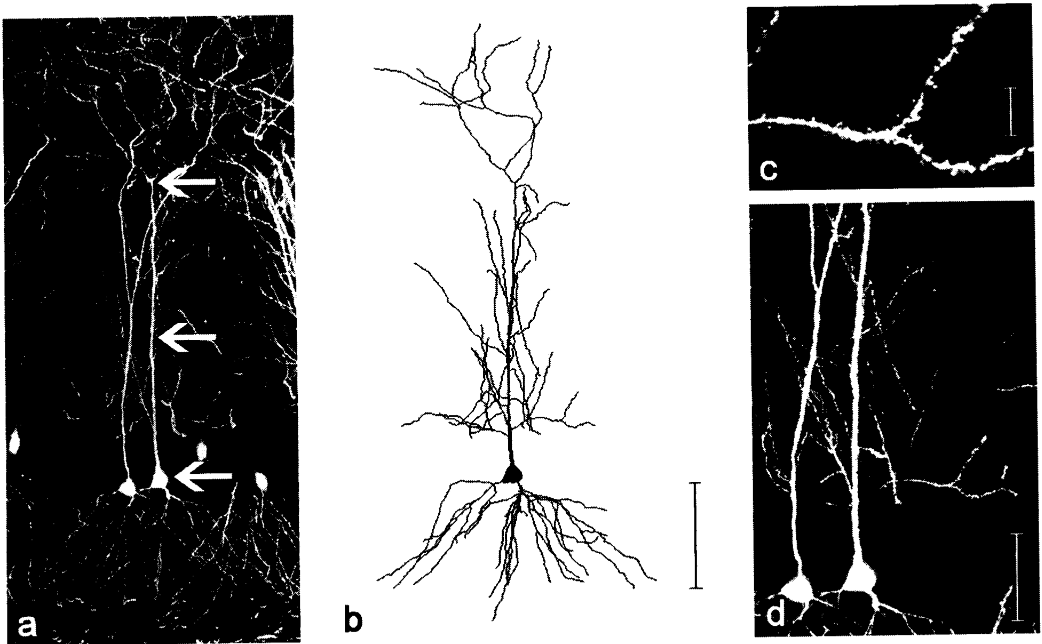

Figure 8.1
Pyramidal neurons from the CA1 region of the mouse dorsal hippocampus. (a) A stacked series of confocal images of CA1 pyramidal neurons expressing green fluorescent protein in a slice from an adult transgenic male mouse (Feng et al., 2000). (b) Computer-generated reconstruction of the pyramidal neuron indicated by arrows in a and shown at the same scale as a. Scale bar, 100 μm. (c) and (d) Higher magnification images of dendritic trees illustrated in a. (c) Tertiary dendrite with bifurcation at the arrow. The location of this dendrite within the tree is indicated by a corresponding arrow in d. Scale bar, 10 μm. (d) Stacked series of confocal images of the two neurons shown in a. Only a subset of the images used in a is included in the stack here to illustrate that individual dendrites of interest can be traced without interference from other labeled neurons when the number of scans in the stack is reduced. The arrow indicates the bifurcating dendrite shown in c. Scale bar, 50 μm.

architecture, a peroxidase-linked antibody to GFP can be used with silver staining techniques in semithin sections (Tyrer et al., 2000). This method has the advantage of viewing GFP-labeled neurons or processes in context with major anatomical landmarks, such as commissures, axon tracts, and neuropils.

Suggestions for Avoiding Artifacts Introduced during Histological Processing of Tissue

The protocols required for staining single neurons for subsequent reconstruction can easily destroy the value of the tissue itself. Injection of biocytin or cobalt chloride (especially), long recording times, or hypoxia can introduce structural artifacts (including blebbing that may easily be mistaken for synaptic varicosities). Such capricious, nonuniform distortions can never be adequately compensated for during recon-

struction and must be eliminated through refinement of injection and histological protocols.

Dehydration of the stained and fixed tissue through an ascending alcohol series, followed by "clearing" in a reagent such as methyl salicylate or other mounting media inevitably leads to overall shrinkage of the preparation. Shrinkage can be as large as 50% with some standard protocols. Any shrinkage will result in a systematic underestimate of morphological parameters. As described earlier, dehydration in glycerol reduces shrinkage to a minimum but can still result in some artifacts.

A practical approach toward monitoring and compensating for any residual artifacts that cannot be eliminated through an optimization of histological protocols is as follows: (1) Use a fluorescent marker in conjunction with the dye used for staining. (2) After injecting the cell with both dyes and before the tissue is put through any histological procedure that might introduce distortion, record an image of the neuron with a fluorescence microscope at high enough magnification to resolve the diameters and relative position of dendritic branches at several positions in the cell. (Note that it is safest to make exposures after a light paraformaldehyde fixation, since illumination of dye-filled cells can result in photoactivated damage and artifactual blebbing.) (3) Record the corresponding images after the complete histological protocol. Shrinkage can then be measured directly and used to obtain a set of scaling factors for correction of model parameters.

One method that has proved successful uses a filling solution with a combination of biocytin and carboxy-fluorescene in the recording electrode. An image of the neuron is obtained immediately following an experiment, using a scanning laser microscope. The confocal microscope is optimal for this purpose since high-resolution, high-magnification images of dendrites relatively deep in unfixed tissue can be obtained, and the net illumination can be kept low enough to avoid photodamage and consequent morphological distortion. Alternatively, tissue can be lightly fixed (i.e., 5 min in 4% paraformaldehyde) and observed with conventional fluorescence microscopy. After recording of several sample images, the tissue is fixed and incubated with HRP-conjugated avidin. The avidin–biotin–HRP complex is subsequently processed through the standard DAB reaction protocols to yield a densely stained cell.

Selection Criteria for Choosing Cells to Reconstruct

To obtain the best results from reconstructions of stained neurons, selection criteria should be used in choosing cells for further analysis. Neurons with filling artifacts such as swollen processes, or faintly stained distal processes, should not be used for reconstructions. Only uniform labeling will facilitate the digitization process and the accuracy of the resulting reconstruction. After selecting a well-labeled neuron, the investigator must consider the issue of cut branches. Although invertebrate neurons

that are labeled in intact ganglia do not exhibit cut processes, the slicing procedures needed to prepare vertebrate tissue invariably result in severed dendrites. If a neuron has been stained in the animal and the tissue sectioned afterward (such as occurs with GFP-expressing neurons or in vivo injections), then it may be possible to locate the remaining portion of a cut dendrite in an adjacent section. However, if a neuron is labeled in a slice (such as with biocytin injections in in vitro preparations), then it is quite likely that cut dendrites will be present. To deal with this problem, most investigators develop criteria specifying a maximum number of cut dendrites and their locations. For example, one might specify that a labeled cortical pyramidal neuron will be reconstructed if (1) none of the primary apical dendrites are severed; (2) none of the primary basal dendrites are cut within 100 µm of the soma; and (3) there are ten or fewer cut dendrites on the entire neuron. The criteria are usually devised in accordance with the objectives of the experiment and should take into account previous data on the cell type of interest. An alternative approach to compensate for the cut dendrites is described in chapter 9.

8.2 Methods for Obtaining Anatomical Data at the Ultrastructural Level

For modeling studies where accurate measurements of dendrites, spines, synapses, and subcellar organelles are required, serial section transmission electron microscopy (ssTEM) is the method of choice. The resolution of ssTEM is needed to distinguish and measure dendrites, axons, glial processes, synapses, and subcellular organelles such as synaptic vesicles, polyribosomes, and endosomal compartments. ssTEM is a labor-intensive enterprise; hence, choosing when to use it is important. Confocal and multiphoton microscopy provide lower-resolution images from living cells to assess whether labeled structures have changed location or size; however, the resolution is too low to identify, quantify, and measure the dimensions and composition of structures in the brain neuropil. ssTEM is required for accurate identification and measurement of objects smaller than 250 nm. High-voltage electron tomography coupled with manual segmentation of cellular structures also produces excellent results, although the size of the sample area is much less than with ssTEM, and this approach still requires time-intensive reconstruction and analysis (Soto et al., 1994; Sosinsky et al., 2005).

Another method has recently been added to the electron microscopy toolkit: serial block-face scanning electron microscopy (Briggman and Denk, 2006). This approach has the advantage that fragile serial thin sections need not be handled; instead, the remaining block face is photographed after each section is removed. Theoretically, larger sample areas could be photographed without montaging; however, the resolution achieved at the lower magnification does not allow deciphering of individual

axons, astroglial processes, dendritic spines, synapses, and the composition of organelles in brain neuropil.

Transmission electron microscopy (TEM) usually requires that sections through biological specimens be less than 100 nm thick. These thin sections allow electrons to pass, being stopped only where stains delineate objects of interest. A sample area of 500–1,000 μm^3 spanning 250–500 serial thin sections is practical with ssTEM. Here we describe methods optimized to produce uniform ribbons of serial thin sections. The goal is to produce sections of perfectly uniform thickness along fold-free ribbons for accurate quantitative analyses. In Harris et al. (2006) we describe detailed methods for obtaining large numbers of ultrathin serial sections for 3-D reconstructions of dendrites, spines, and other structures at high resolution. The main steps are briefly described here and a detailed protocol can be found in the original paper.

Tissue Preparation

Rapid fixation of the tissue is essential for the preservation of ultrastructure and can be achieved either through perfusion of the animal or immersion of the tissue in fixative combined with brief microwave irradiation with aldehyde fixation (Jensen and Harris, 1989). This last method produces excellent results and is especially good for fixation of brain slices. Slices of brain tissue (200 μm thick) are immersed in aldehyde fixative (6% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer with 2 mM $CaCl_2$ and 4 mM $MgSO_4$) and irradiated briefly (10 s) in a microwave oven, restricting final temperature to less than 40 °C. The fixed slice may be processed immediately or stored overnight in fixative at room temperature. The next day, the slices are first rinsed in cacodylate and then transferred to 0.1 M phosphate buffer and vibra-sliced to 70 μm. Storage of the 70 μm vibra-slices in buffer for more than a day can have detrimental effects on tissue fixation because the fixative is diluted out. The result is a blurring of membranes and darkening of cytoplasm.

Microwave-enhanced staining and dehydration followed by manual resin infiltration appear to be optimal from the perspective of saving time and achieving complete infiltration of the resins. Vibra-slices surrounding the region of interest are postfixed in reduced osmium (1% OsO_4/2% KFeCN in 0.1 M cacodylate) for 5 min, rinsed in cacodylate buffer, and transferred to 1% OsO_4 in 0.1 M cacodylate buffer in the microwave at 175 W under vacuum with the microwave on for 1 min, off for 1 min, and then on again for a total of 3 min. Sections are then dehydrated at 250 W under vacuum through a series of ethanol dilutions, with all dilutions containing 1% uranyl acetate (UA) to enhance polyribosome staining. Infiltration with epoxy resins is done outside the microwave oven on a rotator in 1:1 and 1:2 ethanol to propylene oxide (PO) for 10 min each; two changes of 100% PO for 15 min each; 1:1 PO:epoxy resin for 1 hr, and 1:2 PO:epoxy resin overnight. The next morning the blocks are transferred through three changes of 100% epoxy resin for 1 hr each and then

properly oriented in coffin molds with fresh 100% epoxy resin and cured for 48 hr in a conventional oven at 60 °C. All mixtures with epoxy resins contain the 2,4,6-tri-dimethylaminemethyl phenol (DMP-30) accelerator. These procedures produce epoxy blocks of uniform infiltration and sufficient hardness to obtain thousands of serial ultrathin sections.

Serial Section Techniques

A section thickness of 45–50 nm is optimal for minimizing overlap between small structures, such as synaptic vesicles (~30–60 nm) or narrow axonal or astroglial processes (~50 nm) that would be obscured by neighboring structures in thicker sections. Care should be taken to start with a block shaved into a trapezoid with the following shape: a height of about 30 μm, a width of about 100–200 μm, and a depth of 20–30 μm for stability and with one side slanted for orientation. Ribbons of serial thin sections are cut and then retrieved on Pioloform-coated slot grids. Lowicryl and some other resins are notoriously difficult to ribbon because the hydrophilic sections fall apart; however, a few quick sprays of salon-quality hair spray and overnight drying produce uniform continuous ribbons on the same trapezoid.

Using a 35-degree diamond knife will minimize the compression of sections. The ribbon will curve if the north and south edges of the trapezoid are not parallel. Uneven thickness of sections is easily corrected by enclosing the ultramicrotome to prevent air drafts and local temperature changes. Stopping and restarting the ribbon can also produce a change in section thickness. A video camera mounted near the microtome can be used to monitor progress. It is essential not to open the enclosure doors until the desired ribbon length has been achieved. These methods produce long, fold-free, and clean ribbons of serial thin sections with a uniform section thickness. Updates on these methods can be found at http://synapse-web.org, along with detailed methods of photography, alignment, reconstruction, and measurement in three dimensions.

8.3 Reconstruction Techniques for Cells from Whole Mounts and Confocal Images

To date, the most accurate way of obtaining high-quality 3-D reconstructions is to manually trace the structure of the neuron using software programs developed for that purpose (Capowski, 1985; Jacobs and Nevin, 1991), and the most widely used of these programs is the Neurolucida system (Glaser and Glaser, 1990; Microbrightfield, Colchester, VT; http://www.mbfbioscience.com/neurolucida). The branching structure of the neuron is captured as a finite set of points that represent the 3-D coordinates, diameter, and connectivity to other points in the reconstruction. This vector-based format is easy to store, share, and archive, and contains all of the infor-

mation about the geometry of the neuron. Neurons digitized in this format can be used for morphometric analyses as well as modeling studies.

There are two basic techniques used to reconstruct neurons at the light microscopy level: direct tracing from whole-mount preparations in which the entire cell is contained in a thick section or a ganglion, or tracing from a series of confocal images of a fluorescently labeled cell.

Whole-Mount Reconstruction Techniques

Neurons labeled with any of the compounds that result in a dense product, including biocytin, biotinylated dextran amine, cobalt chloride, or the Golgi method, can be digitized directly from whole mounts using a computer–microscope system.

Overall, manual tracing in 3-D is a time-consuming, subjective process. The accuracy and consistency of the reconstruction process depends on the individual collecting the data. Regardless of the functionality of the software or the reconstruction system itself (mechanical stages, microscope, etc.), the quality of the reconstruction depends on how rigorous the operator is when encoding the dimensions of the structure. When neurons are digitized directly from whole mounts, most investigators have found that a 60× or 63× objective provides sufficient resolution for tracing fine neuronal processes and has a working distance that allows successful imaging of processes located toward the middle of the slice (Rihn and Claiborne, 1990).

For complex vertebrate neurons, it is best to digitize the cell body first and then each major dendrite that exits from the soma. Although there are conversion programs (described later) that will convert the digitized neuron into isopotential compartments, care should be taken to ensure that each digitized segment does not change in diameter along its length. Datapoints should be taken at least every 5 μm and at additional points to capture branch points and curves in the branches (Claiborne, 1992). The diameter of the cursor used to trace each branch should be matched to the diameter of the dendrite as closely as possible, but where it is difficult to obtain accurate diameter measurements for the thinner dendrites, measurements taken at the ultrastructural level can be used to correct the diameter measurements. The Neurolucida system allows the operator to label specific parameters, including branch points, natural terminations of neuronal processes, and dendritic spines. In addition, when an entire neuron is not captured in a single thick section, the severed dendrites at the edge of one section should be marked as such in the data file. If additional sections are available, they can be lined up with the severed ends of the same processes. These labeled dendrites can then be digitized and added to the tree.

Similar techniques can be used to digitize invertebrate neurons, with the caveat that some of these cells have much larger and more complex dendritic trees than do hippocampal pyramidal neurons or cerebellar Purkinje cells, with reconstructions containing up to 10,000 segments compared with 1,500–3,000 for vertebrate neurons

(Jacobs and Theunissen, 1996, 2000). In previous studies, using a reconstruction system designed in the Jacobs lab (Jacobs and Nevin, 1991), two different techniques for reconstructing large interneurons and sensory neurons were developed. For interneurons, the cell body was digitized first, followed by the major branch points along the primary neurite to the axon. When a termination point was entered, the software would automatically move to the last open termination point and the subsequent branches could be digitized. Thus each major branch could be digitized in reverse order, from the axon back to the cell body. This approach is very useful in that as long as the operator does not miss any branch points, the software program will keep track of all open termination points, thus ensuring that no major portions of the tree are missed. These reconstructions took up to 50 hr to complete in some cases.

A simpler method can be used to digitize parts of the neuronal structure. For example, in studies where the number, distribution, and location of (synaptic) varicosities were measured and modeled (Jacobs and Nevin, 1991; Jacobs and Theunissen, 1996, 2000), the primary branching structures of the sensory afferent axonal terminals were digitized and the varicosities entered as individually labeled points. These reconstructions captured the main branching structure of the axon terminal and the distribution of varicosities. These partial reconstructions can be completed in approximately 3 hr, a huge savings of time compared with doing a complete reconstruction. Although these reconstructions cannot be used to model the electronic properties of these cells, the location, size, type, and distribution of the varicosities can be used to model synaptic contacts onto postsynaptic neurons (Jacobs and Theunissen, 2000; Cummins et al., 2003).

Reconstructions of Neurons from Confocal Images

Recent developments in both commercial (Neurolucida) and open-source software (Neuron_Morpho, described later) now provide methods for reconstructing fluorescently stained neurons from stacks (Z series) of confocal images (K. M. Brown et al., 2005). A series of images can be obtained from the preparation, stored, and reconstructed offline. Previous attempts to reconstruct fluorescently labeled cells directly from thick tissue sections as described here have been less successful, owing to fading of the label under long illumination times. Digitizing directly from a stack of confocal images avoids bleaching the dye and allows the reconstruction to be performed on any computer. This can be done either with the Neurolucida software using the confocal module or with the Neuron_Morpho software application described later.

For neurons labeled with fluorescent dyes such as DiI or GFP, a series of overlapping stacks of images (with 0.5-μm steps between scans) are acquired with a confocal laser scanning microscope using a 40× objective. Each stack covers a portion of the tree, and although the exact amount of overlap needed will depend on the qualities of

the objective, an overlap of about 50 μm is usually sufficient. The number of image stacks will depend on the size of the dendritic tree; for example, hippocampal pyramidal neurons in mice usually require three to five stacks. The stacks are first montaged together and then the entire dendritic tree is digitized. Because of the resolution of the confocal images and the computer monitor, it can be difficult to obtain accurate diameter measurements for the thinner dendrites. A sampling of dendritic diameters can be measured with the confocal microscope, followed by correction of the data files (O'Boyle et al., 1993). When labeled neurons have dendrites extending into adjacent sections, the severed dendrites at the edge of one section can be marked in the data file and then lined up with the cut ends of the same processes in the next section.

Giorgio Ascoli and his colleagues have developed an open-source tool *Neuron _Morpho* (K. M. Brown et al. 2005; http://www.personal.soton.ac.uk/dales/morpho/) that allows the accurate reconstruction of neurons from confocal image stacks. This tool works as a plug-in for the popular ImageJ (http://rsb.info.nih.gov/ij/) open-source tool developed and distributed by the National Institutes of Health (Bailer, 2003). Both of these tools are free and are compatible with all major computer platforms (Windows, Mac, and Linux). Neuron_Morpho takes advantage of the familiar functionality of ImageJ image handling, yet provides mouse-actuated tracing of dendritic processes. Another useful feature is the "return" function that is activated when an end point to a dendritic branch is entered. The program automatically scans back to the most recent branch point.

First, a set of tiled optical sections that encompass the entire extent of the dendritic tree is collected and then merged into a single dataset using VIAS (Volume Integration and Alignment System; http://www.mssm.edu/cnic/tools.html), a freeware tool that automates the alignment of confocal images (Rodriquez et al., 2003). The merged image stacks are then loaded into ImageJ for manual reconstruction and the files converted into the standard format, Stockley-Wheal-Cannon (SWC) (described later) for import into either NEURON or GENESIS modeling programs (see the software appendix). The reconstruction can be calibrated by imaging a calibration grid and measuring it using the length tool in ImageJ.

A comparison of these tools with Neurolucida (using the confocal module) demonstrated no loss in accuracy and only a small difference in ease of use (K. M. Brown et al., 2005). Of interest, however, was the observation that neurons reconstructed using Neuron_Morpho have many more segments than the same neurons reconstructed using Neurolucida, although both programs produce reconstructions of similar accuracy. This may be due to the differences in the digitization process: in Neuron _Morpho, segments are delineated with vertical lines drawn on either side, whereas in Neurolucida, a circular cursor is used to mark the boundaries.

General Sources of Measurement Error in Light-Microscopic Measurements of Cell Dimensions

Regardless of the method used to make the reconstruction, there are several significant intrinsic practical limitations to light microscopic measurements of neuronal structures. The finest dendritic processes, with diameters less than about 0.5 µm, cannot be detected adequately for reconstruction and will be missed. Fine processes between about 0.5 and 2 µm can be resolved, but the diameters cannot be measured accurately with conventional (wide field) light microscopy. In practice, owing to the characteristic point spread function of a particular optical system, such fine branches will appear larger in diameter than their true size. Very fine in-foldings and undulations in dendritic processes cannot be resolved, leading to a systematic underestimate of dendritic surface area.

Although these problems would seem to be very serious, their effects upon simulated cell responses can be estimated and corrected to a large extent. The key, as mentioned earlier, is to make high-resolution measurements of sample areas within the tissue using a confocal microscope, or preferably, using correlated light and electron microscopy. For example, the high-resolution images can be used to measure the density and morphological parameters of spines, varicosities, and/or in-foldings in dendritic segments of a range of different diameters and locations. These true values can be compared with the low-resolution values extracted through a light microscopic analysis to obtain systematic correction factors, which can be applied to the anatomical data before computer model parameters are set. For example, cortical neurons can be labeled and reconstructed for dendritic measurements and then samples of dendritic segments can be re-embedded for electron microscopy (Megías et al., 2001). Accurate spine density measurements taken from serial sections then can be incorporated into the resulting data files and computational models.

Another approach is to estimate and quantify the impact of these errors by running simulations of the model when several orders of very fine dendritic branches are added onto or deleted from the model's structure. The extent to which such operations change a simulated response depends on the type of question asked by the simulation. If one is calculating the complex input impedance at a point on a fine branch of a dendrite, and one adds or subtracts extra branches near that point, then the calculations could change significantly. If, however, one is calculating the complex transfer impedance between the bases of two different second-order dendritic branches, then the addition or deletion of several fifth- or sixth-order branches has an insignificant effect.

The same general approach can be used for errors in surface area measurements that are due to subresolution of fine structure: errors are estimated, correction factors are used, and the model's tolerance is estimated by bracketing simulations. The

errors of the second type given here (i.e., the misrepresentation of process diameters in the 1–2-μm diameter range) are more problematic. Using confocal microscopy and reconstructing the cell from image stacks as described earlier could minimize these errors. Dendritic processes could be reconstructed down to the 2-μm level, which is well above the level of ambiguity for a typical confocal scope. All processes below 2 μm could be dealt with statistically from electron microscope observations (see Megías et al., 2001).

Ultrastructure Reconstruction Techniques

Serial section electron microscopy provides the best resolution for neuronal structure at both the cellular and subcellular levels. A suite of open-source software tools, *Reconstruct* (Fiala, 2005; http://synapse-web.org/), has been developed by Fiala and Harris (2002) to aid the manual segmentation of neuronal structures of interest from serial electron microscope sections. Many microscopy studies require reconstruction from serial sections, a method of analysis that is sometimes difficult and time-consuming. When each section is cut, mounted, and imaged separately, section images must be montaged and realigned to accurately analyze and visualize the three-dimensional structure. Reconstruct is a free editor designed to facilitate montaging, alignment, analysis, and visualization of serial sections (figure 8.2 shows the interface for Reconstruct). The methods used by Reconstruct for organizing, transforming, and displaying data enable the analysis of series with large numbers of sections and images over a large range of magnifications by making efficient use of computer memory.

The alignment of images from sections with some types of nonlinear deformations, including cracks and folds, can be corrected, although when sections are handled carefully as described in Harris et al. (2006), folds and cracks can be avoided. A large number of different structures can be easily traced and placed together in a single 3-D scene that can be animated or saved. A number of other software packages are available to support structure-oriented reconstruction from serial sections, but do not provide the full functionality of Reconstruct. Some of these commercial programs (e.g., 3-D Doctor, Amira, Neurolucida) provide algorithms for automatic alignment of an entire stack of sections. Automatic alignment plug-ins are also available for the public domain image-processing package ImageJ. However, the quality of these "autoalign" functions needs to be carefully monitored before using them for reconstruction.

An excellent detailed tutorial and documentation of the Reconstruct program has been written by John Fiala. There are six basic steps to this process, described briefly here:

1. First the operator creates a series by designating a directory where the files for the reconstruction will be stored.

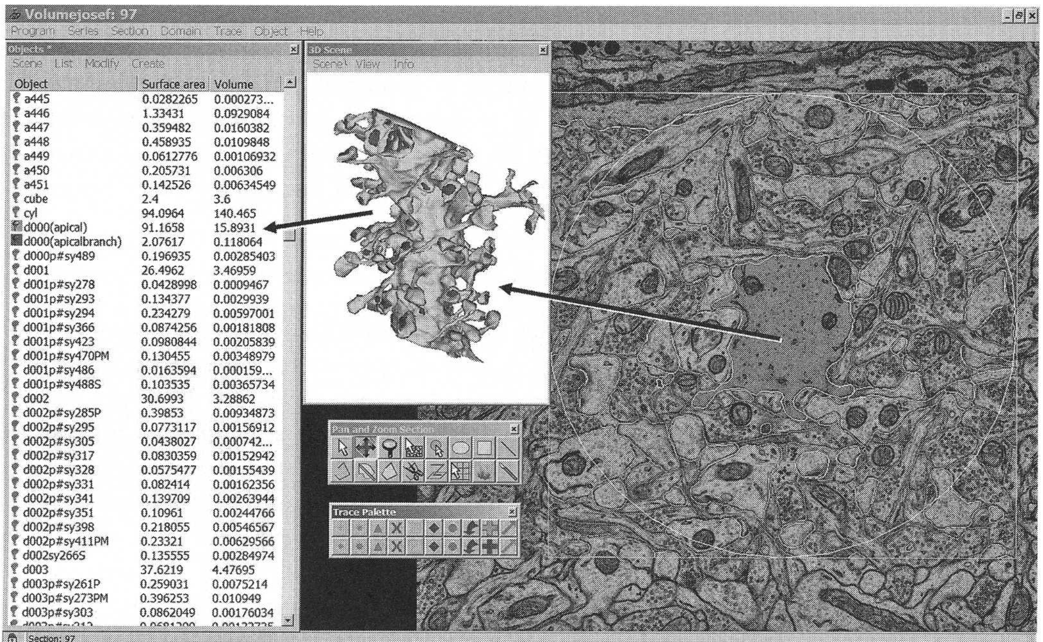

Figure 8.2
User interface for the Reconstruct software program. The left part of the image shows quantitative data for each of the traced contours in the reconstruction. The center field shows a section with traced profiles and the center filled profile is a section of the reconstructed dendrite shown to the left.

2. Then a set of sections are imported as image files in formats that include Windows bitmaps, GIF, TIFF, and/or JPEG files. Each image file should contain only a single image (not an image stack) and represent one section in a series of eight or twenty-four bits per pixel.

3. The next step is to calibrate the pixel size to specify the size of the image picture elements (pixels) in terms of the units of measure of the series. In most cases the pixel size should be adjusted by using the image of a calibration grid or scale that was photographed with the series. Section thickness should also be calibrated using the cylindrical diameters method (Fiala and Harris, 2001).

4. Then the sections in the series must be aligned. Tissue that has been physically sectioned and then imaged must be realigned for accurate 3-D measurements and visualizations. Section images can be manually moved into relative alignment using keyboard commands; however, the software supports a semiautomatic method where the operator specifies correspondence points in two adjacent sections and the program aligns them to each other.

5. The next step is to trace the profiles of the structures of interest within the sections. The operator may chose to reconstruct a single dendrite or organelle, or specify multiple objects and create an ensemble reconstruction showing the relationships between neuronal structures within the scene. Contours are drawn with a pencil tool and filled with a color when the contour is closed. The process is repeated throughout the series of images.

6. At any time during the reconstruction, the operator can view the 3-D object that is being traced. Objects can be viewed in any combination and the scene window supports rotation, pan, and zoom. The objects may be viewed in wire frame or surfaced using a Boissonnat surfacing algorithm. These scenes can be saved for publication as bitmaps or as virtual reality modeling language (VRML) objects for additional smoothing, lighting, and rendering in other 3-D programs (e.g., DReAMM, 3-D Studio Max, Amira etc).

The software can be obtained from the http://synapse-web.org site, which also provides tutorials, extensive documentation, and numerous examples of reconstructed dendrites, spines, and subcelluar structures.

3-D Reconstruction of Cellular Ultrastructure Using Electron Tomography

High-voltage electron tomography is an alternative method that can be used to obtain reconstructions of subcellular anatomy. This method produces a series of images that can be manually segmented using a combination of commercial and open-source software tools. Mark Ellisman's laboratory at the National Center for Microscopy and Imaging Research (NCMIR) has pioneered the development of these tools. The methods for preparing samples, tomographic imaging, and alignment of images are described in depth in a recent modeling study on the node of Ranvier (Sosinsky et al., 2005) that uses the anatomical data obtained through a tomographic reconstruction as the basis for a simulation study using the MCell modeling environment (see the software appendix). Examples of tomographic data, including all of the data used in the node of Ranvier study, can be viewed and or obtained from the Cell Centered Database (see section 8.5).

Investigators at NCMIR have developed a set of techniques and an extensive set of tools for 3-D reconstruction of cellular components from tomograms. These software tools are available for free (http://ncmir.ucsd.edu/downloads/software/index.shtm). The following is a brief description of these techniques and the tools used.

Features of interest (membrane regions, organelles, and cytoskeletal elements) can be traced from the tomographic data using *Xvoxtrace* (Soto et al., 1994) and rendered using the SYNU 3-D graphics package (Hessler et al., 1992). Xvoxtrace enables the user to use manual tracing techniques for volume segmentation of

tomographic data. Using Xvoxtrace, one can outline features on individual planes of the volume while being guided by simultaneous views of the tracing displayed on a volume rendering or tilt series. Once the contours are traced, they can be viewed using Dend or used to generate surfaces for viewing in SYNU. SYNU works with polygon meshes, stacks of contour lines, or 3-D volumes from confocal Z-series, tomography, or simulations. Graphical representations that combine volume and surface renderings can be computed and visualized using the commercial program Amira (TGS Inc., San Diego, CA; http://www.amiravis.com/). Anatomical data obtained through reconstruction of tomographic images can be imported into DReAMM and used with MCell for modeling studies (see the software appendix).

8.4 Using Accurate Neuronal Morphology in Neuron Models

Chapters 10–11 describe how the structural parameters captured with 3-D reconstruction techniques are combined with biophysical properties to create a model. However, once the reconstruction is complete, the file containing the anatomical parameters must be transformed into formats compatible with modeling platforms such as GENESIS or NEURON (see the software appendix). There are several software tools that provide this functionality: Neurolucida, CVapp (R. C. Cannon et al., 1998), L-measure (Ascoli et al., 2001b), and MIEN.

The Neurolucida system contains an analysis module called *NeuroExplorer*, which has an extensive set of functions for visualizing, editing, and performing morphometric analyses on the reconstructed cells. The program supports 3-D visualization and morphometric analysis and can be used to dynamically rotate, scale, and set the display characteristics of any Neurolucida data file. NeuroExplorer can be used to edit the reconstruction and correct errors in digitizing prior to converting the file into a format for modeling. NeuroExplorer exports files only in the Neurolucida format.

The computational neuroscience community has developed several open-source tools that have much of the same functionality as the Neurolucida system, but are specifically designed to edit and convert the morphological information for use in modeling programs. Two of these programs, CVapp and L-Measure, have been in wide use for some time. MIEN is a new addition to the toolbox. These software suites and their attributes are described in the following paragraphs.

CVapp (R. C. Cannon et al., 1998; http://www.compneuro.org/CDROM/docs/cvapp.html) written in Java, is a visualization, format conversion, and manual editor for digital reconstructions that can convert files from either the Neurolucida or SWC format into either the NEURON or GENESIS format for modeling. The SWC format, developed by Robert Cannon, is a parsimonious format widely used for neuronal

morphologies. Each point is represented by seven numbers corresponding to its unique identity; neuritic type (soma, dendrite, axon); X, Y, and Z position; radius; and the identity of its parent in the path from the soma. CVapp supports a number of editing and viewing functions: error checking, shrinkage correction, and adding or deleting branches. It supports multiple viewing styles and can be run through the web as an applet or installed on the user's machine.

L-Measure, developed by Giorgio Ascoli and his colleagues (Ascoli et al., 2001; http://krasnow.gmu.edu/L-Neuron/L-Neuron/home.htm) allows researchers to extract quantitative morphological measurements from neuronal reconstructions. This tool has some of the same file conversion and editing functionality of CVapp and will convert files from Neurolucida and Neuron_Morpho into the SWC standard format. Given the idiosyncratic, error-prone nature of the digitizing process, L-Measure has been used as a tool to standardize 3-D reconstructions from many investigators. This tool recognizes and fixes irregularities in the reconstruction and flags others for manual inspection and repair. L-Measure also contains a powerful, comprehensive suite of morphometric analysis tools that can be applied to any reconstruction for the quantitative analysis of neuronal structure. Characteristics such as numbers of compartments or branches, total dendritic length, surface area, volume, and a wealth of branching characteristics can be calculated with this tool. Both CVapp and L-Measure have become essential tools for converting "imperfect" 3-D reconstructions into models as well as the quantitative analysis of neuronal structure.

MIEN (Model Interaction Environment for Neuroscience), developed by Graham Cummins (http://mien.sourceforge.net), provides a framework for storing, integrating, and interacting with neuroscience data, including data on anatomy and physiology, abstract mathematical models, and detailed compartmental models. MIEN is not a compartmental model simulator, but it provides an interface to the NEURON simulator for evaluation of compartmental models. MIEN supports editing and display of 3-D reconstructions in the Cell Viewer (figure 8.3). This module provides fully 3-D interactive representations of cells, fiducial marks, synapses, density clouds, and annotations. The viewer uses OpenGL functions to produce quality 3-D images and display them in full 3-D and can simulate the optical sections taken by a microscope with a particular viewing angle and depth of field. It allows user interaction and provides functions for selecting points, sections, paths, and other regions within an anatomical model. The viewer also allows the creation, deletion, and modification of structures and provides tools for spatial alignment (coregistration) of different anatomical datasets using fiducial landmarks or direct measurements of relative size and location. It can also load physiological data and display properties like channel density and membrane voltage by coloring the displayed cells.

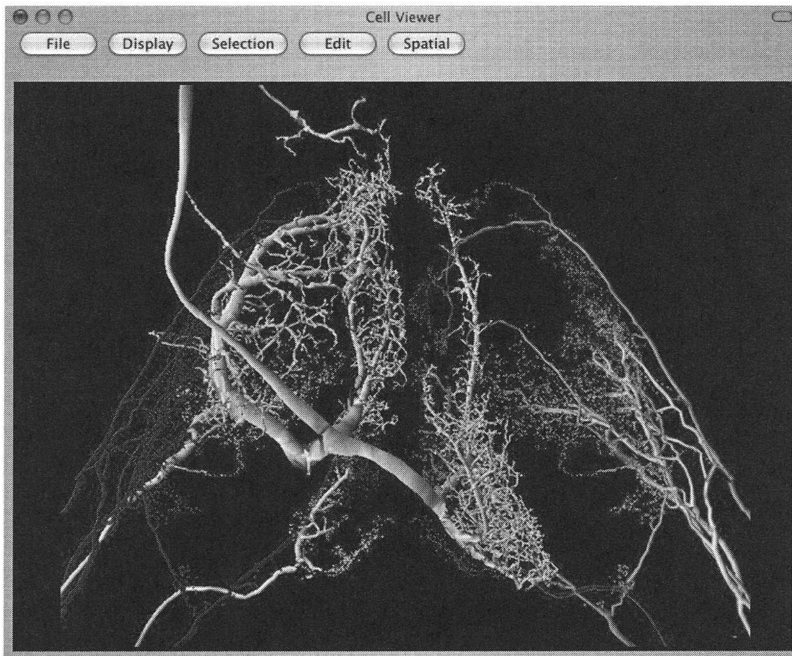

Figure 8.3
Cell Viewer interface from the MIEN suite of software tools. The display shows a cricket interneuron and several mechanosensory afferents that have been scaled and aligned to the same coordinate system. The cells were reconstructed in 3-D and visualized using tools within Cell Viewer.

8.5 Databases and Archives of Neuronal Morphology

Over the past five years, a number of databases and archives of high-quality structural data for the neuroscience community have become available (Ascoli, 2006). Many of these efforts have blossomed through the efforts of investigators supported by the Human Brain Project (Koslow and Hirsch, 2004; Gardner and Shepherd, 2004), and the outcome of this work has produced two comprehensive clearing houses for this type of data as well as many other kinds of neuroscience data: The Neuroscience Database Gateway (NDG) (http://ndg.sfn.org, 2004) and more recently, the Neuroscience Information Framework (NIF) (http://www.neuinfo.org/). The goal of these efforts is to support and enhance data sharing within the neuroscience community. Although there are many existing collections of anatomical data residing in the laboratories of individual investigators, several community databases, which host anatomical data from many different labs, have now been developed. Three of these efforts have emerged as leaders within the neuroinformatics community as providers of data, tools, and ontologies. These three archives are

described in detail here; additional collections can be found at the NDG and in the future at the NIF.

Cell Centered Database

The Cell Centered Database (CCDB) (http://ccdb.ucsd.edu/) was the first web-accessible database for cellular imaging data (Martone et al., 2002, 2003). This database houses structural and protein distribution information derived from confocal, multiphoton, and electron microscopy, including correlated microscopy. The CCDB is affiliated with the National Center for Microscopy and Imaging Research (http://ncmir.ucsd.edu), a national research resource specializing in high-voltage electron microscopy, electron tomography, and the development of state-of-the-art 3-D imaging and analysis technologies for structure and function analysis. The CCDB hosts a wide variety of structural information, including images of many cell types and 3-D reconstructions of individual cells, which are stored as series of optical slices, both with and without deconvolution, and also as branching tree structures traced using Neurolucida.

Datasets obtained with electron tomography are stored along with all of the raw images and the processing details required to reconstruct the volume from the raw data. Each object segmented from the 3-D volume is stored as a separate object indexed to the parent reconstruction. Four types of segmented objects are modeled in the CCDB: (1) surface objects, polygonal surface meshes representing 3-D objects in the reconstruction extracted using either isosurfacing methods or manual contouring; (2) contour objects, a series of contours that have not been fitted with a surface; (3) volume objects, subvolumes containing an object of interest; and (4) tree objects, skeletons of branching objects like dendrites and axons derived from Neurolucida. Each object is stored with such measurements as surface area, volume, length, number, and labeling intensity. The CCDB is an excellent resource for many different types of structural data and advanced microscopy tools and techniques, and serves as a testbed for the development of ontologies for neuroscience and data mining techniques.

NeuroMorpho.Org

This effort, led by Giorgio Ascoli and his colleagues (http://neuromorpho.org), under the auspices of the Neuroscience Information Framework, is a searchable archive of over a thousand digital reconstructions contributed by investigators in other laboratories. More than twenty-two different laboratories have contributed cells from four or more species of animals, and links are provided to the contributors' websites. At least eight identified cell types are represented, with the majority coming from the hippocampus and neocortex. Each neuron in NeuroMorpho.Org is represented by a unique identifier, general information (metadata), the original and standardized

ASCII files of the digital morphological reconstruction, and a set of morphometric features. Each of these reconstructions has been evaluated and edited using the L-Neuron software. Errors and inconsistencies have been corrected, and all reconstructions are stored in the same format: the standard SWC mentioned earlier. This important and growing database serves as the largest curated archive of 3-D reconstructions and represents a huge resource for the computational neuroscience community.

Synapse Web

Synapse Web (http://synapse-web.org/) is a resource developed for understanding and studying the 3-D ultrastructure of the brain. It combines tutorials on the subcellular structure of neurons and comprehensive descriptions of procedures and tools for tissue preparation and 3-D reconstruction (described earlier), an atlas of neurocytology (developed by Josef Spacek), several volumes of aligned serial section images of neuropil in the hippocampus, and numerous fully reconstructed dendrites, some with an electron microscope series of images. This resource provides a set of very high quality educational resources, superb tools for reconstruction, and access to high-quality ultrastructural data.