Optical Imaging of Neural Structure and Physiology: Confocal Fluorescence Microscopy in Live Brain Slices

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I. Introduction

A. Mapping Neural Organization at the Cellular and Synaptic Levels

Understanding brain function, both in the normal and in the diseased state, requires a thorough knowledge of the anatomical and physiological substrates. This necessitates a mapping of the organization and interconnection of populations of neurons, but must also include an intimate understanding of the physiological workings of the individual neural elements, both neurons and glia, since neural function ultimately depends on the organization of synaptic connections at the cellular and subcellular levels (Shepherd, 1998).

A major goal in neurobiology is to provide a detailed map of the anatomical connections between individual neurons and groups of neurons. This should include not only a “wiring diagram” of connections within and between brain regions, but also a qualitative and quantitative description of the subcellular distribution of synaptic contacts. Such a map certainly would prove useful for understanding neural circuitry at a network level. But even an exhaustive map of neuronal synaptic connectivity is not sufficient for figuring out how the anatomy subserves neural function. Information about the physiological properties of the synaptic contacts (e.g., strength, sign, and number) and their consequences for neuronal targets is also needed.
Even from an anatomical standpoint, a single static map of connections may not accurately reflect neural connectivity, especially during development and in diseased states in which physical connections may be changing. Such phenomena fall into the realm of modifiability, or plasticity, and there is now a major effort to understand how structural plasticity may subserve functional plasticity in the developing, adult, and diseased brain. Neural organization can thus be considered to be in a dynamic state, especially at the subcellular level, and we are in essence challenged with mapping an actively changing terrain! Such considerations highlight a need for time-resolved microanatomy as well as a conjoining of anatomical and physiological observations at the network, cellular, and subcellular levels.

Given that neural organization is in a dynamic state, it is important not only to generate a functional map of the brain, but also to elucidate the principles and mechanisms governing the development and plasticity of neural organization. It is anticipated that principles of neural organization will be elucidated by studying the process of construction of network connections during ontogeny. However, spatiotemporal changes in neural organization are at their highest during development, presenting developmental brain cartographers with a most challenging task.

B. The Problem: Cellular Diversity and Complexity of Neural Tissues

A major obstacle to mapping the anatomical substrates of neural function is the complexity of neural organization. Axonal and dendritic processes of neurons have very elaborate shapes, and each cell type, by definition, has unique morphological and physiological characteristics. Moreover, even within a rather homogeneous population of cells, it seems likely that no two cells have exactly the same morphology and pattern of connectivity. In the central nervous system (CNS), axonal processes often take complex paths to reach target regions and, once there, can ramify profusely. Axonal branches can also innervate multiple target regions. Likewise, dendritic branches are typically highly branched and can be recipient to tens of thousands of axonal synaptic contacts.

Over the past century, neuroanatomists have made tremendous strides toward mapping basic neuronal structure and connectivity, largely due to the extensive application of the Golgi technique (e.g., Ramón y Cajal, 1911). The Golgi stain generates a dense reaction product within most or all of the intracellular volume of individual neurons, and since it labels only a small percentage of cells in any given tissue volume, it provides a good method for examining the complex structure of individual neurons at the light microscope level. However, the Golgi technique is limited by unpredictable staining patterns such that the neuroanatomist has little control over the number and type of cells labeled. Furthermore, it is applicable only to postmortem tissue and is therefore of limited value for studies of a physiologic nature.

With the advent of techniques for injecting cells with a tracer dye while making electrophysiological recordings, it became possible to determine in detail the anatomical features of neurons following physiological characterization. This was an important step in efforts to correlate cellular physiology and morphology in brain tissue. Moreover, neuronal tracers such as horseradish peroxidase (HRP) afford the collection of three-dimensional (3D) information on the structure of neurons at both the light and the electron microscope (EM) level (e.g., Deitch et al., 1991). HRP can be injected into tissue to label individual neurons or populations of neurons, but it is visible in the light microscope only after fixation and enzymatic reaction. Consequently, it is not a useful marker for assessing the structure of live cells. Fluorescent tracer dyes such as Lucifer yellow (LY), on the other hand, permit light and electron microscopic observations and, by virtue of their fluorescence, are visible in living cells. In the case of LY, staining for EM is accomplished by HRP immunohistochemistry using antibodies against the LY (Taghert et al., 1982; Holt, 1989). A number of studies have used LY microinjection in conjunction with confocal imaging to examine the 3D structure of neurons in both living (Smith et al., 1991, 1994; Turner et al., 1991, 1993, 1994) and fixed (Belichenko et al., 1992, 1994a,b; Belichenko and Dahlström, 1994a,b, 1995a,b; Trommald et al., 1995) brain tissues. However, two major drawbacks to using LY are that it requires the tedious process of microinjection into single cells and, for EM-level analysis, the cytoplasm of the labeled cell is obliterated by the immunohistochemical reaction. Moreover, these methods of labeling do not directly reveal synaptic contacts and they have not been shown to be useful for directly imaging changes in cellular structure over time.

C. One Solution: Vital Fluorescent Labeling and 3D Confocal Imaging in Brain Slices

Much can be learned about the functional organization of the brain by correlating single-cell physiological analyses with static images of cellular morphology. However, functional mapping dictates that we integrate the structural and physiological features of individual cells into the larger context of tissue organization. This can be facilitated by simultaneously viewing the functional interrelationships and interactions of many cells within organized networks. Live brain slice preparations provide an outstanding opportunity to assess the dynamic structural and physiological features of cells, at high spatial resolution, within complex threedimensional tissue environments.

In the past, it has been necessary to “reconstruct” neural structures and cellular relationships from the microscopic...
examination of several adjacent, relatively thin tissue sections. This is because conventional (both light and electron) microscopic techniques did not provide sufficiently high spatial resolution of cellular and subcellular structures in thick tissue specimens. Although it is still necessary in most cases to section whole brain for microscopic examination, the development and application of modern confocal microscopy (White et al., 1987; Fine et al., 1988; Lichtman, 1994; Dailey et al., 1999) and, more recently, multiphoton microscopy (Denk et al., 1990, 1994) have provided a means to examine structures at high spatial resolution in much thicker (>100 μm) tissue slices. The advantages afforded by confocal microscopy are derived from its ability to collect optical sections of a thick tissue specimen while rejecting light from out-of-focus components of the specimen (Wilson, 1990; Pawley, 1995). These features have made confocal imaging an indispensable tool for analysis of neural organization at the subcellular, cellular, and tissue levels of organization (Turner et al., 1996). Indeed, the confocal microscope was initially conceived and developed with the goal of elucidating the neural organization of the brain (Minsky, 1961, 1988).

The successful application of modern optical techniques to elucidate neural organization has depended on the availability of suitable markers of cellular structure and physiology. Among the most widely used markers are the vital fluorescent membrane dyes for labeling cell surfaces and the fluorescent Ca²⁺ indicator dyes. More recently, it has been possible to express fluorescent proteins or fusion proteins in neurons and glia in order to visualize specific cellular structures such as synapses. This chapter discusses the use of these fluorescent probes and molecular markers in conjunction with high-resolution optical imaging with the confocal microscope to examine the dynamic anatomy and physiology of live brain tissues. It concentrates on the application of these methods to study neural organization at a cellular and subcellular level in tissue slices from developing rodents. However, several of these methods are more widely applicable to studies of normal and diseased adult brain tissues, both pre- and postmortem, as studies from other groups have shown (see references for a sample).

II. Live Brain Slice Preparation and Culture

Live brain slice preparations have been used extensively to study fundamental physiological properties of neurons and local neural circuits using both electrophysiological and optical approaches and, more recently, for examining dynamic morphological features of neurons and glia. The primary advantages of tissue slice preparations are their greater optical and physiological accessibility over in vivo conditions (Pozzo-Miller et al., 1993) and the maintenance of structural and functional integrity of intrinsic synaptic connections vis-à-vis dissociated cell culture preparations. The major disadvantages are: (1) possible structural and physiological alterations (damage) to the tissues as a consequence of the isolation procedure; (2) loss of extrinsic neuronal connections such that the isolated tissues cease to exhibit normal functionality (i.e., the tissues are no longer integrated in the larger scheme of brain function); and (3) a finite period of time before tissue rundown (generally, a maximum of several hours). These drawbacks notwithstanding, the significant advantage that in vitro brain slice preparations hold for optical accessibility has driven the development of slice imaging methodology. Consequently, progress on the development of sensitive fluorescent-light microscopy, new fluorescent probes of cellular anatomy and physiology, and inexpensive computing capabilities has provided a powerful set of tools for investigating the organization and function of neural tissues. Moreover, good methods are now available for maintaining healthy, live brain slices in vitro for long periods of time. This section describes methods currently used for preparing, maintaining, labeling, and imaging live slices of developing rodent brain.

Isolated tissues derived from immature animals generally fare much better than adult tissues. In the case of the rat hippocampus, tissues taken from animals older than about 1 week of age generally do not remain healthy for longer than a few hours in vitro. It seems likely that this limitation is due, in part, to a developmental shift from anaerobic- to aerobic-based tissue metabolism. Moreover, developing brain tissues seem to suffer less stress under the hypoxic and hypoglycemic conditions that may occur during the tissue isolation procedures. Indeed, if brain tissues are isolated from developing animals, they can be maintained in vitro for long periods of time (weeks to months), extending well beyond the corresponding time point from which tissues can be maintained ex vivo when taken from older (>P7) animals. Consequently, many studies of live mammalian brain tissues have employed slice cultures derived from immature brain (see Gähwiler et al., 1997).

There are two common methods for maintaining brain slices in vitro (Gähwiler et al., 1997). One method is based on the so-called “roller tube” culture technique of Gähwiler (1984; Gähwiler et al., 1991). This technique provides a convenient way of mounting tissue slices on glass coverslips for labeling, long-term culturing, and optical imaging. Another slice culture method (Stoppini et al., 1991) in which slices are grown on porous filter membranes also has proven to be very suitable for long-term culture. Both culture methods (Fig. 1) involve rapidly removing the tissues of interest, then slicing the tissues with a tissue chopper (Stoelting, Chicago, IL) or a Vibratome at a thickness of 300 to 500 μm. In the case of roller tube cultures, the brain slices are then secured to alcohol-cleaned glass coverslips (11 × 22 mm) with a mixture of chicken plasma (10 μl; Cocalico) and bovine...
thrombin (10 µl; Sigma, St. Louis, MO). Tissues are adherent within about 10 min, at which point the coverslips are placed in a test tube with 1 ml of Hepes-buffered growth medium containing 25% serum. The tubes are kept in a roller drum tilted at 5° with respect to horizontal. This roller tube method provides constant gentle rolling to aerate the tissue slices, and viable slices with an organotypic tissue organization can be maintained in vitro for up to several weeks. However, the slices thin appreciably over time (down to about 100 µm thick within 1–2 weeks in vitro), and there can be a significant proliferation of glial cells (Dailey and Waite, 1999). The roller tube cultures are especially useful for high-resolution microscopy since the slices are adherent to—a stable glass coverslip (Terasaki and Dailey, 1995; Dailey, 1999).

Slice cultures grown on filter membranes (Stoppini et al., 1991) provide a means for short- or long-term culturing of brain tissues without the need to physically rotate the tissues or attach them to coverslips. Tissue slices are prepared as described above, then placed on cell culture inserts (Falcon 3090 or 3102) containing polyethylene terephthalate, track-etched porous membranes (1 µm pore size). The inserts are placed in six-well tissue culture plates, and culture medium (1 ml containing 50% MEM, 25% Hanks’ balanced salt solution, 25% horse serum, 2 mM glutamine, and 0.044% NaHCO₃) is added to each well. The filter cultures are maintained in an incubator containing warmed (36°C), humidified air (5% CO₂). Translucent culture inserts are used to facilitate periodic inspection of the cultured tissues under a dissecting microscope.

Each tissue slice culture technique has its advantages and disadvantages. In terms of culture preparation, the filter cultures require less effort because there is no need for lengthy processing of coverslips. Moreover, once the slices are situated on the membranes, the tissues can be placed immediately into the incubator without concern for whether the slices are firmly attached to the culture substratum. Some investigators report that tissues cultured by the filter membrane technique show reduced gliosis in comparison to those cultured by the roller tube technique (del Rio et al., 1991), suggesting that long-term cultured brain tissues may fare better on membranes. On the other hand, the roller tube cultures are more easily mounted for microscopic viewing, since the slices are securely attached directly to a piece of glass that can serve as the microscope coverslip (Terasaki and Dailey, 1995). For live tissue imaging, a more complicated scheme is required for mounting tissues in a way that permits on-stage perfusion of solutions (Dailey, 1999).

**III. Labeling Neuronal and Glial Cells in Brain Tissue Slices**

**A. Visualizing Neural Structure with Fluorescent Membrane Dyes**

To examine the structure of neurons in tissue slices, we have used a family of long carbon-chain, carbocyanine dyes (see Table 1), which incorporate into and diffuse laterally within plasma membranes of neurons (Honig and Hume, 1986; Honig, 1993) and other cells that come into contact with the dye. The rapid and complete surface labeling is especially useful for determining the morphology of cells such as neurons that have very long and elaborate branching processes. Consequently, membrane dyes have found widespread use as markers of axonal projections (Baker and Reese, 1993).
A useful property of these membrane dyes is that they can label cells in both living and formaldehyde-fixed tissues. This is because formaldehyde fixation does not extensively cross-link lipids, so that fluorescent lipids can intercalate into the surface membrane and diffuse freely within the plane of the membrane. However, a form of DiI that is formaldehyde fixable is also available (Molecular Probes, Eugene, OR; Catalog No. D7000).

The diffusion rate of the most commonly used fluorescent lipid, DiI, is reported to be 6 mm per day in living tissue (Product Sheet MP282; Molecular Probes). In live brain slices, adequate levels of staining can be achieved in neuronal processes over 1 mm away from the labeling site within a few hours. We have successfully imaged both DiI- and DiO-labeled axons for several hours, but some workers have suggested that live cells labeled with DiO remain somewhat healthier during imaging than those stained with DiI. Perhaps this is because DiI may stain living cells more strongly than DiO (MP282; Molecular Probes).

Often it is most useful to label only a small percentage of the total number of cells within a tissue volume because even the best optical microscopes are unable to resolve the details of fine axonal and dendritic processes when all the tissue elements are stained with the same dye. In certain cases it is desirable to label a select subset of cells. For neural tissue, labeling a select population of cells can often be accomplished simply by varying the location of the dye application. Cell bodies of projection neurons can be back-labeled by injecting a tracer into target regions. Alternatively, axonal projections can be labeled by applying dye to the region of cell bodies, to dendrites, or along known axonal tracts. However, we have found that surface labeling with the membrane dyes (e.g., DiO) seems to be more efficient in the anterograde versus the retrograde direction along axons in living tissue (Dailey and Smith, 1993). This may be related to axonal transport or membrane trafficking patterns in neurons.

We have used several different methods for introducing membrane dyes into tissue slices. One approach involves pressure injection of a solution of dye. A stock solution (~0.5%) of dye is made in N,N-dimethylformamide, dimethyl sulfoxide, or vegetable oil and is injected through a glass micropipette (2–3 µm tip diameter) using a Picospritzer (General Valve). Small, localized injections can be made by presenting a series of brief pressure pulses (1 ms duration, 80 psi) to the back of the pipette.

Another membrane dye-labeling method works very well with relatively thin (50–100 µm) tissue slices, such as those carried as roller tube cultures for longer than 1 week. To label cells, the tip of a glass pipette is dipped into a saturated solution of DiI in ethanol, then inserted, and either removed after a time or broken off within the tissue. The dye solution dries onto the surface of the pipette, leaving a coating of dye crystals that contacts and labels cell membranes when inserted into the tissue. This method seems to produce labeling of fewer but more brightly stained cells with less granular background labeling of neighboring cells. Both the injection and the crystal insertion labeling protocols, when applied to roller tube cultures, benefit from the fact that the plasma clot holds the tissue slice in place during the labeling procedure.

There has been considerable interest in fluorescent markers whose excitation and emission spectra are in the red or near infrared. Although one potential drawback of the longer wavelength red light (vis-à-vis blue or green light) is that the spatial resolution is reduced somewhat, it is expected that optical imaging in tissues will be improved with red dyes because: (1) there is less background auto-fluorescence from tissues at the longer wavelengths; (2) biological tissues should diffract red light less, thereby improving the collection of light for image formation; and (3) the red light is of lower energy than UV, blue, and green light and therefore should produce less photodynamic damage (see later). There may not always be a dye available

### Table 1 Common Fluorescent Membrane Dyes for Assessing Neuronal Structure and Mapping Axonal Projections

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Abs max ((\lambda))</th>
<th>E M max ((\lambda))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DiIC(_{18}(3))</td>
<td>DiI</td>
<td>550 nm</td>
<td>565 nm</td>
<td>Honig and Hume (1986)</td>
</tr>
<tr>
<td>DiI(\Delta_{9,12}C_{18}(3))</td>
<td>Fast DiI</td>
<td>549 nm</td>
<td>563 nm</td>
<td></td>
</tr>
<tr>
<td>DiOC(_{18}(3))</td>
<td>DiO</td>
<td>484 nm</td>
<td>501 nm</td>
<td>Honig and Hume (1986)</td>
</tr>
<tr>
<td>DiIC(_{18}(5))</td>
<td>DiD</td>
<td>644 nm</td>
<td>663 nm</td>
<td>Agmon et al. (1995)</td>
</tr>
<tr>
<td>4-Di-16-ASP</td>
<td>DiA</td>
<td>491 nm</td>
<td>613 nm</td>
<td>Mendelowitz et al. (1992)</td>
</tr>
</tbody>
</table>

*These dyes are available from Molecular Probes, Inc. (Eugene, OR). Data are from Molecular Probes' catalog and information sheets.

Absorption maximum.

Emission maximum.
with suitable spectra, but in the case of the long-carbon-tail fluorescent membrane dyes, there is a longer-wavelength version of DiI, known as DiD, which has a five-carbon linking bridge (see Table 1). The excitation/emission maxima of DiD are 644/663 nm (in methanol), compared to 550/565 nm for the three-carbon bridge of the classic DiI (MP282; Molecular Probes). Thus, laser lines from the argon–krypton (647 nm) or helium–neon (633 nm) lasers can be used to excite DiD. A study by Agmon et al. (1995) indicated that DiD is, in fact, superior to DiI for examining axonal projections in brain slices by confocal microscopy.

A new and potentially powerful approach to obtain Golgi-like labeling of neuronal and glial cells with fluorescent lipids has been described (Gan et al., 2000). This technique, referred to as “DiOlistics,” is a modification of a biologists approach to gene transfection (see later; Lo et al., 1994). Tiny gold or tungsten particles (0.4–1.7 µm) coated with one or more fluorescent lipid dyes (such as DiO, DiI, or DiD) are propelled into brain tissues using a “gene gun” (Bio-Rad, Hercules, CA). Cells whose surfaces are contacted by dye-coated particles become labeled. In the case of neurons, the entire axonal and dendritic arbors, including synaptic spines, are labeled. The DiOlistics approach works in both live and paraformaldehyde-fixed brain tissues, including postmortem tissues derived from human brain (Gan et al., 2000). In live tissues, cell labeling is very rapid, with complete labeling of complex dendritic arbors reportedly occurring within 5 min (apparent diffusion coefficient 10⁻⁵ cm²/s). Labeling is severalfold slower in fixed tissues, consistent with many previous studies utilizing fluorescent lipophilic dyes. The number of labeled cells can be varied by altering parameters such as the density of particles in the “bullets,” the pressure and distance at which the tissues are shot, and the number of shots fired at the tissues. Using particles coated with different combinations of various dyes with different fluorescent spectra, it is possible to label and distinguish many neurons within a small tissue region. For example, using combinations of three different lipophilic dyes, Gan et al. (2000) could distinguish individual cells labeled in one of seven different spectral patterns. Using three-dimensional multichannel confocal fluorescence imaging (see later), it is possible to identify processes from single cells even in densely labeled portions of complex brain tissues. This multicolor labeling methodology thus offers a new approach to mapping the complex organization and relationship of neuronal and glial cells in brain tissues.

Finally, it should be noted that fluorescent lipophilic dyes are useful for morphological studies of neural tissue at both the light and the electron microscope level. This is because the fluorescence emission can convert (i.e., photoconvert) diaminobenzidine (DAB) to an oxidized, electron-dense reaction product that is deposited locally near the fluorophore (Sandell and Masland, 1988). Thus, in the case of membrane dyes that label the plasmalemma, one can produce a nice outline of the cell surface that preserves the intracellular structure of labeled cells. This method could prove to be very useful for determining the ultrastructural characteristics, including the synaptic connectivity, of specific populations of neurons that have been selectively labeled with a fluorescent dye (e.g., von Barheldt et al., 1990; Gan et al., 1999). Moreover, because membrane dyes can be used as vital stains, it is now possible to observe cells in the living state with the light microscope and, subsequently, to examine the very same cells in the electron microscope. This should facilitate studies of neuronal structure and connectivity.

### B. Immunofluorescent Labeling

Antibodies provide a very powerful means for labeling specific populations of cells and subcellular structures in neural tissues, although their use is usually limited to fixed specimens. Sometimes it is desirable to assess the 3D organization of immunostained structures, and optical imaging of immunolabeled thick brain slices provides a relatively convenient means for doing this, so long as the antibodies are able to penetrate the tissues adequately and clear optical sections of sufficient resolution can be collected. Immunohistochemical staining of thick brain slices has been shown to be feasible, and confocal microscopy affords collection of the 3D immunofluorescence data with high spatial resolution (e.g., Vincent et al., 1991; Welsh et al., 1991).

As an example, we have used antibodies against synaptic proteins in acutely isolated and in cultured brain slices (Dailey et al., 1994; Qin et al., 2001). One such antibody, generated against the protein synapsin-I (syn-I), has been shown to be specific to nerve terminals (DeCamilli et al., 1983a,b) where it is associated with small synaptic vesicles (Navone et al., 1984). Immunostaining of hippocampal slices (100–400 µm thick) with syn-I antiserum is performed after light fixation (2% formaldehyde for 10 min) and a rigorous extraction process (1% Triton X-100 for 24 to 72 h) (Dailey et al., 1994). Penetration of the rather large antibody proteins into the thick tissues appears to be a major limitation. Therefore, we (1) perform the membrane permeabilization step on a rotating stage to provide constant mechanical agitation (~100 rpm) and (2) lengthen the primary and secondary antibody incubation times to several hours or overnight (4°C). This staining procedure provides a sufficient immunofluorescent signal to image small synaptic structures as much as 50 to 100 µm deep into a brain slice. The availability of a wide spectrum of fluorescent probes as well as confocal imaging systems with multiple laser lines permits double- and triple-label immunohistochemical analyses of brain tissues (Sergent, 1994; Wouterlood et al., 1998). As indicated earlier, the use of longer wavelength fluorophores, such as Cy5 (650/667 nm) (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) or members of the Alexa Fluor series [Alexa Fluor-633 (632/647 nm), Alexa...
Fluor-647 (650/668 nm), or Alexa Fluor-660 (663/690 nm), from Molecular Probes), may improve the detectable fluorescence signal from deeper portions of the specimen.

Immunohistochecmical staining also may be combined with staining by fluorescent membrane dyes (Elberger and Honig, 1990). These methods even permit immunohistochemistry in combination with time-lapse observations of live cells in brain slices. For example, O’Rourke et al. (1992) followed Dil-labeled migrating neuroblasts in slices of developing cerebral cortex by time-lapse confocal microscopy and then fixed the slices and immunohistochemically stained the tissues with an antiserum to reveal radial glial fibers. They first photoconverted the DiI to permanently mark the labeled cells with a stable, electron-dense DAB reaction product and then permeabilized the tissues for antibody staining.

C. Gene Transfection and Expression of Fluorescent Proteins

We have seen that fluorescent membrane dyes provide a means for assessing gross cellular morphology and that immunohistochemical methods afford more specific labeling of synaptic structures in fixed preparations. However, the complexity of neural tissues often makes it difficult to map the organization of synaptic structures in relation to cellular structure. One promising approach is to use molecular genetic tools to label synaptic structures within individual cells or defined populations of cells. The exploitation of a jellyfish green fluorescent protein (GFP) as a reporter of gene expression (Chalfie et al., 1994), and more recently as a marker of protein distribution in cells, has opened the possibility of visualizing specific cell populations and cellular structures in both living and fixed brain tissues. Indeed, GFP and related fluorescent proteins (Tien, 1998; Tsien and Prasher, 1998) are increasingly being used to mark neurons and glia in brain tissues (e.g., Lo et al., 1994; Moriyoshi et al., 1996; Zhuo et al., 1997; Vasquez et al., 1998; van den Pol and Ghosh, 1998; Chamberlin et al., 1998).

GFP expression in neurons and glia can be used in a variety of ways to facilitate mapping of brain anatomy and microstructure. Expression of soluble GFP fills the entire extent of neurons and glia, and this can be used to define cellular anatomy. Modification of the GFP to target it specifically to the plasma membrane (Moriyoshi et al., 1996; Tamamaki et al., 2000) may facilitate lateral movement in long processes and thus provide better results for anatomical studies in neurons. Under control of cell-type- or region-specific regulatory genes, GFP can be used as a reporter system to label specific neuronal or glial cell populations (e.g., Zhuo et al., 1997; Oliva et al., 2000; Spergel et al., 2001). Alternatively, GFP may be fused to proteins of interest containing specific cellular targeting sequences. For example, GFP fused to a synaptic protein results in specific labeling of pre- or postsynaptic structures in transfected neurons (e.g., Arnold and Clapham, 1999; Ahmari et al., 2000). This offers the exciting possibility of mapping specific synaptic structures at the single-cell level within brain tissues.

A variety of approaches have been used to introduce foreign genes such as GFP and GFP-fusion proteins into cells and tissue slices in vitro. These include viral constructs for infection (Vasquez et al., 1998) as well as nonviral transfection methodologies such as particle-mediated biolistics (Lo et al., 1994), liposome-mediated transfection (Murphy and Messer, 2001), and single-cell electroporation (Haas et al., 2001). For transfection experiments in brain slices (Marrs et al., 2001; Qin et al., 2001), we utilize the biolistics approach (Fig. 2) based on the methods of Lo et al. (1994). A Helios gene gun (Bio-Rad) is used following the manufacturer’s instructions. Colloidal gold particles (1 µm diameter, 8 mg) are combined with 0.05 M spermidine (100 µl; Sigma), 1 M CaCl2 (100 µl), and DNA (15–20 µg). Hippocampal slices are shot (2–3 mm, 70–80 psi) between 5 and 10 days in culture and then quickly returned to the incubator. Slices are fixed (4% formaldehyde in culture medium, 10 min, 4°C) 1–2 days later. In some cases, antibody staining is performed on tissues following fixation.

D. Ca2+-Sensitive Fluorescent Probes for Studying Neuronal and Glial Physiology

There are now numerous probes for addressing questions of cellular physiology (Mason, 1993; Yuste et al., 1999a). These include both fluorescent and nonfluorescent probes of intracellular calcium, magnesium, protons, sodium, zinc, and several other physiologically important molecules. Some of these probes are ratiometric and provide estimates of absolute concentrations of the molecular species of interest.

The use of voltage-sensitive dyes has played an important role in mapping patterns of neural activity and in the organization of synaptic connections in isolated tissue slices (e.g., Grinvald et al., 1988). However, fluorescent calcium-sensitive dyes (e.g., fura-2 and fluo-3) (Gryniewicz et al., 1985; Minta et al., 1989) have been used more widely to study the physiological properties of neural and glial cells as well as the physiological organization of developing and mature nervous system tissues. The fluorescent calcium probes are especially useful because large changes in intracellular calcium are associated with neural electrical activity (Ross, 1989), and it is clear that intracellular calcium is an important second messenger associated with a wide variety of neuronal functions (Kennedy, 1989; Ghosh and Greenberg, 1995). An early study by Yuste et al. (1992) showed the power of Ca2+ imaging in isolated brain tissue slices for determining features of cellular communication in the developing cerebral cortex. Several studies since have utilized Ca2+ imaging in semi-intact brain tissues to study...
In the case of dye labeling ("DiOlistics"; Gan et al., 1994), particles carrying various combinations of fluorescent dyes can be used to differentially label cells. A transmitted light (differential interference contrast, DIC) image and fluorescence image (Fluor) of the same cell are shown 1 day after being shot with plasmid DNA encoding the GFP. Note the DNA-bearing gold particle (arrowhead in DIC image) located within the cell.

Figure 2 Fluorescent labeling of neurons and glia in brain tissues by gene gun-mediated particle bombardment (biolistics). Tiny (0.4–1.7 µm) gold or tungsten particles are coated either with fluorescent lipophilic dyes (e.g., DiI, DiO) or with plasmid DNA encoding GFP or GFP-fusion proteins. Particles carrying fluorescent dye or DNA are propelled into tissues using a blast of helium. In the case of dye labeling ("DiOlistics"; Gan et al., 2000), cells are labeled when contacted by dye-coated particles. Labeling of axonal and dendritic arbors is very rapid (~5 min) in live tissues due to the diffusion of the lipophilic dye within the plane of the plasma membrane.

Both living and fixed tissue preparations are amenable to DiOlistics labeling. Particles carrying various combinations of fluorescent dyes can be used to differentially label individual cells within tissues. For gene transfection (Lo et al., 1994), particles bearing DNA must penetrate the cell and come to reside within or near the cell nucleus. Gene expression for 12 to 48 h is usually necessary to produce sufficient fluorescent signal. Particles bearing different plasmids [e.g., encoding green (GFP) or red (DsRed) fluorescent proteins] can be used to differentially label cells. A transmitted light (differential interference contrast, DIC) image and fluorescence image (Fluor) of the same cell are shown 1 day after being shot with plasmid DNA encoding the GFP. Note the DNA-bearing gold particle (arrowhead in DIC image) located within the cell.

With regard to staining brain tissue, AM–ester loading works better on slices from embryonic tissues or in slices that have been maintained in culture for a period of time. For example, suitable fluo-3 AM loading of acutely prepared (<8-h-old) CNS tissue slices is difficult to achieve, but slices cultured for a week or two stain robustly. In fact, some success is achieved even after 12 to 24 h in vitro (Dailey and Smith, 1994). It is unclear why staining is enhanced in the cultured slices, but one possibility is that the tissue "loosens up" somewhat. Others have also found extreme difficulty staining acute CNS tissue slices with AM–ester dyes when the tissue was taken from rats older than 10 days of age (Kudo et al., 1989; Yuste and Katz, 1991; O’Donovan et al., 1993). Adams et al. (1994) suggested that the penetration of AM ester dyes into thick brain slices may be facilitated by cleaving some ester bonds prior to labeling.

There are a variety of ways of introducing the physiological probes into neural tissue slices. Perhaps the most reliable is by direct intracellular injection. Microinjection allows one to select individual cells of interest as well as control the concentration of the intracellular dye. This approach has been used successfully to image intracellular calcium at high spatial and temporal resolution within neuronal cell bodies and processes as small as individual dendritic spines (Tank et al., 1988; Müller and Connor, 1991; Guthrie et al., 1991; Svoboda et al., 1996; Yuste et al., 1999b). However, one may want to label a population of identified neurons, or specific axonal projections and synaptic terminals, rather than single isolated cells. This can be achieved by localized superfusion (Regehr and Tank, 1991) or injection (O’Donovan et al., 1993) of dyes into axonal tracts, leading to uptake and anterograde and retrograde transport of the dye. Such an approach is useful so long as the axons are bundled or spatially confined and the dye can be applied close to the site of interest since labeling is limited by intracellular diffusion.

Bulk loading of cells using membrane-permeant dyes provides less specific, more widespread labeling of neurons and glia in tissue slices. This approach makes use of dyes with acetoxymethyl (AM) groups linked to fluorophores by ester bonds. The AM–ester form of the dye is membrane permeant until the ester bonds are cleaved by endogenous esterases within the cells (Tsien, 1981). Once the ester bonds are cleaved, the dye molecule is trapped within the cell where it becomes a useful indicator of cellular physiology.

With regard to staining brain tissue, AM–ester loading works better on slices from embryonic tissues or in slices that have been maintained in culture for a period of time. For example, suitable fluo-3 AM loading of acutely prepared (<8-h-old) CNS tissue slices is difficult to achieve, but slices cultured for a week or two stain robustly. In fact, some success is achieved even after 12 to 24 h in vitro (Dailey and Smith, 1994). It is unclear why staining is enhanced in the cultured slices, but one possibility is that the tissue "loosens up" somewhat. Others have also found extreme difficulty staining acute CNS tissue slices with AM–ester dyes when the tissue was taken from rats older than 10 days of age (Kudo et al., 1989; Yuste and Katz, 1991; O’Donovan et al., 1993). Adams et al. (1994) suggested that the penetration of AM ester dyes into thick brain slices may be facilitated by cleaving some ester bonds prior to labeling.

the development and organization of neural circuits in brain (Dailey and Smith, 1994; Guerineau et al., 1998) and retina (Wong et al., 1998).

In addition to neurons, astrocytes (Cornell-Bell et al., 1990; Dani et al., 1992) and other glial cells (Jahromi et al., 1992; Reist and Smith, 1992; Lev-Ram and Ellisman, 1995) in the nervous system also exhibit sizable intracellular fluctuations in calcium. Moreover, it is well documented that a variety of stimuli can induce waves of calcium activity within glial cell networks. Such trans-glial calcium signals have been proposed to have important roles in long-range cellular signaling within the brain (Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991; Smith, 1994; Charles, 1998). Thus, in generating a "map" of functional networks within the brain, it will be important to consider the organization of both neuronal and nonneuronal tissue components.
IV. Imaging Methodology

A. Confocal Microscopy

The principle of confocal imaging is illustrated in Figure 3. A wide range of confocal imaging systems are now available. We used several different confocal imaging systems in making the observations described here, including three commercially available microscopes and a custom-built confocal microscope. The commercial confocals used were a Bio-Rad MRC-500 (Bio-Rad), modified as described previously (Smith et al., 1990), a Noran Odyssey that was capable of collecting semiconfocal fluorescence images at video rate (30 frames/s), and a three-laser Leica TCS NT confocal system (Leica, Heidelberg, Germany). The custom-built microscope was a relatively low-cost, optical-bench style inverted microscope designed and built by Stephen Smith (Stanford University). For illumination, the microscope was equipped with a 25-mW argon-ion laser (Ion Laser Technology) and a 15-mW helium–neon laser (MWK Lasers). The laser beams were steered though a shutter, neutral density filter wheel, and excitation filter wheel and onto a neutral beam-splitting mirror that reflected 7% of the laser light to the specimen. The filter wheels were operated by stepper motors controlled by the host computer. The neutral beam-splitting mirror, which substituted for the dichroic mirror that is typically used in fluorescence microscopy, permitted single detector imaging of different fluorophores without the need to remove or adjust the beam-splitting mirror. Light was detected through an adjustable circular aperture in front of the detector, a gallium–arsenide photomultiplier tube. This microscope had a very high throughput and was thus well suited to long, time-lapse experiments. The microscope objective lenses we found to be most useful for the custom-built microscope were a dry Nikon 20×/0.75 Fluor, a Zeiss Plan-Neofluor 25×/0.8 (oil–water–glycerin), and an oil-immersion Olympus DApO 40×/1.3 UV objective. For the Leica system, we used a 20×/0.7 dry Plan Apo or a 63×/1.2 water Plan Apo (220 µm working distance) lens.

B. Three-Dimensional Imaging

An important feature of the confocal microscope is its ability to collect three-dimensional information on the structure of complex cells and cellular relationships at high spatial resolution (Lichtman, 1994). With regard to neural organization in the brain, the 3D information obtainable with the confocal microscope is helping to map the relationship of neurons within functional networks (Smith et al., 1991) and localize synaptic structures at both the light (Hosokawa et al., 1992, 1994; Belichenko and Dahlstrom, 1995b) and the EM (Deitch et al., 1991) level of organization.

The question of how best to display the 3D image data is challenging, and the problem is compounded when 3D data are collected over time (generating 4D data sets; see later). For single time-point data sets, each of the individual optical sections along the axial (z) dimension can be displayed separately (e.g., Fig. 11A), or the axial stack of images can be combined to generate a pair of stereo images (e.g., Fig. 10). Stereo-pair images can be generated from any through-focus data set simply by shifting each successive image in the stack a small amount (i.e., by 1 pixel) before combining the images. For each of the stereo-pair image stacks, individual images are shifted in the opposite direction (i.e., shifted left for one stereo-pair image and right for the other). Generally it is best to recombine images using a maximum brightness operation (rather than a simple addition) to reduce the buildup of background noise. The disadvantage of recombining the image stack (either for stereo or for nonstereo viewing) is that adjacent image plans may contain a small amount of out-of-focus light from a given structure, and when images are recombined, this can reduce the sharpness and contrast of the structure. This problem can be effectively defeated in the confocal microscope by stopping down the pinhole aperture in order to reject the maximum

![Figure 3](image-url) The principle of laser scanning confocal fluorescence microscopy. Laser light for fluorescence excitation is focused by a microscope objective lens to a diffraction-limited spot within a thick tissue specimen, such as a brain slice. Fluorescence emission is collected by the same objective lens and is directed via a dichroic or neutral beam-splitting mirror to a photomultiplier tube (detector). Light (dashed line) from regions of the specimen outside the confocal plane is rejected by the pinhole aperture in front of the detector (i.e., only light from a single, narrow focal plane is detected).
amount of out-of-focus light and reduce flare. However, this will substantially decrease the detected signal and is most useful under conditions under which phototoxicity is not a concern and the illumination intensity can be turned up to compensate for the loss of signal.

C. Dynamic Imaging in Live Brain Slices

We have applied confocal imaging technology in conjunction with vital fluorescent labeling to examine dynamic changes in cellular structure and physiology in live brain slices. Some of the important considerations for live brain slice imaging are discussed next.

1. Maintaining Brain Slices on the Microscope Stage

Factors that seem to be critical for maintaining healthy brain slices on the microscope stage include temperature, pH, glucose levels, and oxygenation (Dailey, 1999). Slice physiologists have long known that oxygen deprivation can have severe effects on physiological properties such as synaptic activity, although CNS tissues from developing mammals seem to be fairly resistant to hypoxia (e.g., Dunwiddie, 1981) and hypoglycemia (Crépel et al., 1992).

It is not always easy to assess the health of living tissue on the microscope stage, but in the case of dynamic processes such as cell division, migration, or axon extension, one would expect that the cells perform these activities at rates near that expected based on other methods of determination. Also, one should become suspicious if the rate of activity consistently declined or increased over time when imaged. For example, exposure of fluorescently labeled axons to high light levels can reduce the rate of extension or cause retraction. In contrast, high light levels can produce a long-lasting increase in the frequency of Ca\textsuperscript{2+} spikes in fluo-3-labeled astrocytes in cultured brain slices. In many cases, there will not be a useful benchmark for determining phototoxic effects, but consistent changes during imaging will serve to warn the concerned microscopist. It may be worth sacrificing a few well-labeled preparations to determine if different imaging protocols, such as lower light levels or longer time intervals between images, will significantly alter the biological activity under study.

The specific requirements for maintaining healthy tissues during imaging will dictate specimen chamber design (Dailey, 1999). Two important chamber considerations are whether to superfuse the tissue with bathing medium and whether to use an open or closed chamber. The closed chamber has the advantages of preventing evaporation during long experiments and stabilizing temperature fluctuations. We found that brain slices maintained in closed chambers (volume ~1 ml) with Hepes-buffered culture medium remain viable and vigorous for about 6 h, after which point the chamber medium acidifies and cell motility declines noticeably. However, when the old chamber medium is exchanged with new medium, the cells "jump to life" again. This crude method of periodic medium exchange has supported the continuous observation of DiI-labeled migrating neuroblasts in brain slices on the microscope stage for as long as 45 h (O’Rourke et al., 1992). However, when using this approach, one runs the risk of mechanically disturbing the chamber or inducing a temperature change and thereby causing a jump in focus.

Continuous superfusion provides a more reliable method of medium exchange and introduction of experimental reagents. A variety of perfusion chambers with either open or closed configurations are available (Warner Instruments, Inc., Hamden, CT). Sometimes it is necessary to design and construct very sophisticated temperature and fluid level control systems (e.g., Delbridge et al., 1990). Such chambers may permit a very rapid exchange of media, which is often necessary for physiological experiments requiring a fast exchange of reagents. Programmable automated perfusion systems that permit rapid switching between one of several perfusion channels are available (Warner Instruments or AutoMate Scientific, Inc., Oakland, CA). Some experimental conditions require only relatively simple, low-cost chambers and perfusion systems. We have used an inexpensive, custom-made perfusion system to continuously superfuse tissue slices on the microscope stage for many hours (Fig. 4). The tissue can remain healthy for 20 h or more when perfused (10 to 20 ml/h) with either the culture medium (Dailey
et al., 1994) or normal saline, both of which are buffered with 25 mM Hepes.

Specimen heating may be essential for some experiments, but this can induce an agonizing battle with focus stability (see later) as the chamber and stage components heat up. A sufficient period of preheating can help alleviate some of these problems. A relatively simple heating device can be constructed by modifying a hair dryer to blow warm air onto both the chamber and the stage (Dailey, 1999). It is also important to monitor the temperature of the perfusing medium very near the specimen. Low-cost microprocessor temperature controllers that reduce fluctuations in the heating/cooling cycle are available (Omega Engineering, Stamford, CT).

2. Depth of View

The goal of studies in tissue slices is to examine biological structure and physiology within a complex cellular environment that approximates that found in situ. In the case of live brain slices, it is generally desirable to image as far from cut tissue surfaces as possible to avoid artifacts associated with tissue damage. For example, the cut surfaces of developing brain tissues contain an abnormal cellular arrangement that includes a plethora of astrocytes and microglia, as well as a mat of growing neuronal processes. Although time-lapse imaging of these regions provides striking footage of glial cell movements, proliferation, and phagocytosis (Smith et al., 1990; Dailey and Waite, 1999; Stence et al., 2001), one has to be cautious when drawing conclusions about the normalcy of these events in relation to mature, intact brain.

With oil-immersion lenses, useful fluorescence images seem to be limited to a depth of 50 to 75 µm or so into the tissue. When imaging deep (>50 µm) within tissue, spatial resolution can suffer from several factors, including (1) weak staining of cells due to poor dye penetration; (2) light scatter by the tissue components; and (3) spherical aberration.

The first problem (weak staining) can be overcome if the dye is injected into the tissue with a minimum of disruption. Also, as noted previously, the loading of some dyes may be enhanced by culturing the tissue briefly. Conceivably, light scatter by the tissue can be minimized by using longer wavelength dyes. Imaging at longer wavelengths may also reduce phototoxic effects since the light is of lower energy. Finally, the problem of spherical aberration, which is exacerbated when imaging through various media with differing indices of refraction, is improved by using water-immersion objective lenses (see Pawley, 1995). Fortunately, microscope manufacturers have been responsive to the need for well-corrected water-immersion objective lenses with long working distance and high numerical aperture.

3. Signal-to-Noise and Spatiotemporal Resolution

When imaging any dynamic biological events, there is generally a trade-off between the signal-to-noise ratio ($R_{s/n}$) (which affects the spatial resolution) and the temporal resolution. The demands of high spatial resolution (high $R_{s/n}$) restrict temporal resolution. $R_{s/n}$ is proportional to the total number of photons collected. Thus, for scanning confocal imaging systems, higher $R_{s/n}$ can be achieved by: (a) intensifying the fluorescence staining; (b) increasing the incident illumination; (c) improving the collection efficiency of the system; or (d) increasing the absolute number of photons collected by lengthening the dwell time on a single-pixel, line, or frame-by-frame basis.

A primary concern when imaging live, fluorescently labeled cells is the photon collection efficiency of the optical system. This is especially critical when imaging dynamic processes, such as axon or dendrite extension, over long periods of time. Too much light can quickly halt growth. Systems with a higher collection efficiency will afford lower light levels, thus permitting more frequent sampling or experiments of longer duration before photodamage ensues.

A common means of improving the resolution is to average successive images, or frames. This can certainly increase the $R_{s/n}$, but if cell structure or physiology changes rapidly, frame averaging can “smear” the data in both the spatial and the temporal domains. It may be better to increase pixel dwell time to improve $R_{s/n}$ although this can also induce temporal distortion within single images such that the top and bottom portions of a scanned image are collected several seconds apart (see later discussion).

It should be noted that $R_{s/n}$ will also drop off as one focuses into the thick tissue specimen. Consequently, it is generally necessary to increase the incident (excitation) light to maintain a comparable spatial resolution when collecting three-dimensional data from thick tissue specimens such as brain slices. This problem can be compounded by weaker staining of structures located in deep portions of tissues.

4. Focus Drift

The thin optical sections produced by the confocal microscope reduce out-of-focus flare and improve resolution over standard wide-field optical imaging methods. However, with such a shallow depth of focus, even very small changes in the position of the microscope objective relative to the structures of interest within the specimen can create problems. This is particularly evident when imaging thin, tortuous structures such as axons or fine dendritic processes within live brain slices. A moving focal plane can, for example, give one the deceiving impression of axon or dendrite extension or retraction. This problem is compounded when imaging cells and cell processes that are actively moving within tissues.

One obvious approach is to image the cells in four dimensions (3D × time) (Fig. 5). Such “volume imaging” can keep structures in view in the face of minor tissue or stage movements; it is also helpful for monitoring axonal and dendritic
processes that grow through one focal plane to another. Our strategy has been to image with the detector aperture (confocal pinhole) in an open configuration, which translates into a pinhole size roughly two to four times the Airy Disk. Although this reduces the axial resolution somewhat, it has the dual advantage of achieving a higher signal-to-noise ratio at a given illumination intensity as well as broadening the optical section. On the custom-built microscope, the open pinhole configuration gives an apparent optical section thickness of about 3 µm when using a 40×/1.3 objective. Thus, for each time point, images are collected at axial step intervals of 2 to 3 µm. In the case of the Leica TCS NT system, the axial steps are less than 1 µm when using a 63×/1.2 water lens. The guiding principle here is to space the image planes in the axial dimension to maximize the volume of tissue imaged but not to lose continuity between neighboring optical sections. When stacks of images are collected at 3- to 5-min intervals at power levels of ~50–75 µW (near the back aperture of the objective), DiI- or GFP-labeled cells do not appear to suffer phototoxic effects and can be imaged continuously for over 20 h (40× objective, zoom 2). Image stacks can be later recomposed using a maximum brightness operation (ImageJ; Scion Corp., Frederick, MD) so that segments of thin (<1 µm) axons and dendrites that course through the various z-axis images appear contiguous in a single image. Unfortunately, even when z-axis stacks of images are collected, tissue movements can be so severe as to necessitate a continuous “tweaking” of the focus to keep cells within the stack. Thus, it is helpful to store the recently collected images in such a way that they are quickly accessible and can be reviewed on the fly to make corrective focus adjustments. Alternatively, one can keep track of focus drift by simply marking in-focus features on an acetate sheet taped to the computer monitor. Corrective focus changes can be made as needed during the time-lapse experiment to keep the features within a given image plane.

Ideally, one would like an automated means of maintaining the desired plane of focus, especially for long time-lapse imaging sessions. Although there are several autofocus methods that work for simple specimens, imaging structures in tissues presents significant challenges because there is no single image plane within the specimen on which to calculate focus. One approach is to calculate focus from a stationary reference plane, such as the surface of the coverslip (Ziv and Smith, 1996). This may work as long as the tissue volume of interest maintains a fixed relationship to the coverslip, which is not always the case.

5. Photodynamic Damage

For observations on live, fluorescently labeled cells, the problem of photodamage is generally a limiting factor for achieving high spatial and temporal resolution. A trade-off exists between illumination intensity and resolution. Stronger incident illumination intensities enable faster data collection at a given spatial resolution.

In neural tissues, some of the most obvious signs of phototoxicity include a decrease in intracellular vesicle traffic, axon and dendrite retraction, blebbing of labeled processes and cell bodies, triangulation of dendritic branch points, and sustained rises in intracellular Ca2+.

As discussed earlier, there are theoretical reasons for thinking that use of the longer wavelength dyes, such as DiD, will reduce photodamage. Indeed, it has been reported that DiD seems to cause less collateral damage to living erythrocytes than DiI (Bloom and Webb, 1984). This is consistent with the idea that the longer wavelength light used to excite DiD (i.e., 633 or 647 nm) is of lower energy than the excitation lines for DiI. However, our preliminary time-lapse observations suggest that axonal and dendritic elements in brain slices stained with DiD are more susceptible to photodamage than those stained with Dil. Thus, it is important to carefully assess each dye to determine its properties under the particular experimental conditions under which it will be used.

6. Data Management

Imaging tissues in three dimensions over time can produce very large data sets. For example, collection of 20 optical sections (512 × 512-pixel arrays) at 5-min intervals over a 10-h-long experiment generates 600 Mbytes of image data per channel. It is now feasible and often useful to collect two or more image channels simultaneously (e.g., two fluorescence and one transmitted light channels), generating gigabytes of image data per day. Fortunately, storage and retrieval of mass digital data are not nearly as problematic as they were just a few years ago. For example, recordable compact discs (CDs) offer a highly versatile, cost-effective, and nearly universal means of archiving large amounts of image data. Recordable digital video discs, with a severalfold larger storage capacity, may soon supplant CDs as a common medium of choice for archiving large data sets.
V. Application: Mapping Neural Structure and Physiology in Developing Brain Slices

We have applied the methods for fluorescence labeling and confocal imaging described earlier to study the organization and development of neural systems in isolated brain slices. From an anatomical standpoint, this has included three-dimensional mapping of axonal and dendritic branches, as well as synaptic structures. Dynamic structural changes associated with axonal and dendritic growth and synapse formation are also being examined in order to elucidate principles of developmental plasticity. From a physiological standpoint, we have investigated patterns of neuronal and glial activity in both acutely isolated and organotypically cultured brain slices from developing rodents. These examples serve to demonstrate the spatial and temporal resolution available for mapping neural structure and physiology in semi-intact mammalian brain tissues.

A. Organization and Growth of Axonal Fibers

As a model system, we have examined the organization and development of the hippocampal mossy fiber system. The mossy fibers are the axons of dentate granule cells that synaptically contact pyramidal neurons in area CA3 (Henze et al., 2000). The organization of the mossy fiber projection was examined by staining with vital fluorescent membrane dyes. Injection of DiI or DiO into the dentate gyrus of live hippocampal slices labels within a few hours the full extent of the mossy fibers that project into area CA3. In slices taken from mature animals, one can see individual giant varicosities that are spaced along the length of mossy fibers (Fig. 6). These varicosities likely correspond to the giant presynaptic terminals that impinge on the CA3 pyramidal cell dendrites (Blackstad and Kjaerheim, 1961; Chicurel and Harris, 1992).

Confocal 3D reconstruction was used to examine the three-dimensional organization and development of mossy fiber axons and their giant terminals. Individual fibers were found to have serpentine pathways through the complex tissue environment, and individual synaptic varicosities could be clearly resolved (Fig. 7).

We next used time-lapse imaging to explore the dynamics of mossy fiber growth in developing hippocampal brain slices. Fibers in live tissue slices were labeled with DiI or DiO as described earlier, then imaged over a period of several hours. Single-focal-plane images were collected at 1- or 2-min intervals or, more frequently, stacks of five to seven images were collected at 5-min intervals. Based upon such 4D imaging movies, mossy fiber axons were found to extend within target regions at rates of about 10–30 µm/h (Dailey and Smith, 1993; Dailey et al., 1994). Growth was often saltatory, showing phases of rapid growth that were interrupted by short quiescent periods, and axons reaching the edge of the fiber bundle frequently went through several rounds of retraction and redirection. In some cases, new branches sprouted laterally from the shafts of axons (Fig. 8).

B. Structure and Development of Neuronal Dendrites

Most excitatory synaptic connections in the brain are formed on dendritic projections called spines. We used fluorescence labeling and confocal imaging to address the development and dynamics of neuronal dendrite branches and spines (Dailey and Smith, 1996). To examine dendrites of pyramidal neurons in slices, fluorescent membrane dye is injected near the region of the basal dendrites (Fig. 9).

When dye is injected into slices taken from early postnatal rats, the elaborate organization of neuronal cell bodies and dendritic arbor is revealed. The three-dimensional organization of dendrites is best appreciated in stereo-pair images collected from a tissue volume at a relatively low magnification (Fig. 10). At higher magnification, confocal images reveal the fine microstructure of dendrites. Dendritic branches are studded with numerous filopodia and spine-like protrusions that extend into the surrounding tissue in all directions. These spiny protrusions are sites of synaptic termination by afferent axons.

Time-lapse imaging of fluorescently-labeled dendrites in developing tissue slices demonstrated that dendritic microstructure is quite dynamic (Dailey and Smith, 1996; Marrs et al., 2001). Developing dendritic branches bear a combination of fleeting filopodia-like protrusions and relatively stable spine-like structures (Fig. 11). Since dendritic spines typically correspond to axonal synaptic input, the dynamic changes in spiny structures on developing dendrites may be a morphological correlate of synaptic plasticity. Based on time-lapse imaging, there is now substantial evidence that dynamic dendritic filopodia are precursors to more stable dendritic spines (Ziv and Smith, 1996; Friedman et al., 2000; Marrs et al., 2001). The extent to which dendritic spines and the synaptic structures associated with spines are dynamic in more mature tissues remains an open question (Okabe et al., 1999).

In the future it will be of interest to examine directly the interaction and dynamic changes of pre- and postsynaptic components at identified sites of synaptic contact. This may be facilitated in experiments in which axonal and dendritic processes are labeled with different dyes to facilitate their identification (Fig. 12).

C. Organization of Neural Synapses

Mapping neuronal connections would be simpler if there were unique markers of the cells involved and of the synaptic contacts between each particular set of neurons. Some synaptic systems in the CNS do, in fact, have unique structural
features that facilitate their mapping. The mossy fiber synapses, for example, are characterized by giant (>2 µm) presynaptic terminals and large, complex postsynaptic spines that are readily identifiable even by light microscopy. We have examined the three-dimensional organization of mossy fiber terminals in brain slices using the antibody to syn-1, described earlier. Immunohistochemical staining of thick tissue slices isolated from formaldehyde-fixed rat brain reveals mossy fiber giant synaptic terminals (Fig. 13).

We also have used immunohistochemical techniques to assay the development and organization of mossy fiber synaptic contacts in brain slices cultured for 1–2 weeks.

Figure 6 Dil-labeled mossy fibers in a live brain slice prepared from a 3-week-old rat. (A) Low-magnification view showing site of dye injection (*) near the dentate gyrus, which is the location of the granule cell bodies and the source of the mossy fibers. Note the tight bundle of labeled mossy fibers (arrowhead) that extend from the dentate gyrus into area CA3. Scale bar, 100 µm. (B) High-magnification view showing individual fibers and varicosities. The large, en passant varicosities (arrow) correspond to giant mossy fiber synaptic terminals. Scale bar, 25 µm.
Figure 7 Three-dimensional organization of Dil-labeled mossy fibers in brain slices from developing rat. (A) Stereo-pair images of the mossy fiber bundle (arrow) in a slice from a P5 rat, at a time when the mossy fibers are just growing out and forming synaptic contacts with CA3 pyramidal neurons. Depth of view is 30 µm. (B) Stereo-pair images of mossy fibers in a live slice prepared from a P12 rat. Note the tortuous course of individual axons (arrowhead) within the mossy fiber bundle. The giant en passant varicosities (arrows), corresponding to synaptic terminals, can be seen along the length of the mossy fiber axons. Depth of view is 15 µm. Scale bar, 50 µm for A, 10 µm for B.
Three-dimensional imaging of syn-I-stained tissues revealed that the giant synapses maintain a stereotypical distribution along the apical dendrites of CA3 pyramidal neurons in cultured slices, although individual terminals appear slightly smaller and less complex than their in vivo counterparts (Fig. 13).

Antibodies against postsynaptic proteins also have been developed, and these permit studies of synaptic organization as well (Qin et al., 2001). For example, we have used antibodies against a postsynaptic density protein, PSD95, which is a PDZ-domain scaffold protein that serves to organize and link neurotransmitter receptors to the postsynaptic cytoskeleton (Cho et al., 1992; Kistner et al., 1993; Sheng and Sala, 2001). Immunohistochemistry using the anti-PSD95 antibody in hippocampal tissues highlights regional differences in the organization and size of synaptic structures (Fig. 14). The large and complex postsynaptic structures at mossy fiber synapses are especially evident in the stratum lucidum, where mossy fiber axons normally course, but they are also evident at lower density in the pyramidal cell body layer. In contrast to area CA3, immunostaining in area CA1 shows only small, typical postsynaptic structures.

Although immunohistochemical staining can provide information on the size and density of synaptic structures in brain tissues, they yield little information on the distribution of synaptic structures in relation to individual cells. To address questions of synaptic organization on individual cells, we utilized gene gun transfection and expression of a GFP–PSD95 fusion protein (Qin et al., 2001). To label postsynaptic structures, we used a GW1 vector containing enhanced GFP fused in frame to the C-terminus of PSD95,
Figure 10 Stereo-pair images showing DiD-labeled CA1 pyramidal neurons near a site of dye crystal insertion (*). Note 3D organization of labeled cell bodies and apical dendrites (arrowhead), which branch and course throughout the thickness of the brain slice. In one case, the axon (arrow) can be seen emerging from the pyramidal cell body. Depth of view is 40 µm (20 images at 2-µm-step intervals). Slice is from a P6 rat and cultured for 5 days. Scale bar, 50 µm.

Figure 11 Imaging dendritic spine dynamics in a live brain slice from developing rat. (A) Through-focus series of a portion of a DiD-labeled dendrite from a CA1 pyramidal neuron in a live brain slice (P5, 7 days in vitro). The optical section depth is indicated in micrometers. These images were collected with the confocal pinhole aperture in the fully open configuration to maximize the signal detected. As a result, some out-of-focus flare is evident in the various optical sections. Scale bar, 25 µm. (B) Time-lapse sequence of same field as in A showing dynamics of dendritic spines. These are extended-focus images made by combining six optical sections collected at the depths indicated earlier. Note the shortening of a spine (arrowhead) and transient extension of filopodia-like protrusions (filled and open arrows). Such changes in dendritic structure may reflect plasticity in synaptic function. Time is shown in minutes.
under control of a human cytomegalovirus promoter (gift from D. Bredt, University of California at San Francisco).

Expression of the fusion protein, which targets normally to synapses, labels all synaptic sites in the transfected neurons. Confocal analysis and three-dimensional reconstruction of transfected tissues yield striking images of synaptic localization. Such data can reveal dramatic differences in the organization of synapses, on neighboring cells and even on different portions of the same cell, thus helping to define unique synaptic domains and identify distinct neuronal cell types (Fig. 15).

Notably, cells expressing GFP and GFP-fusion proteins can be examined in the living state, enabling studies on the dynamic formation and remodeling of synaptic structures. Such changes are a consequence of electrical activity in neurons (Ross, 1989; Sinha and Saggau, 1999; Smetters et al., 1999), and glia also have been found to generate intracellular Ca^{2+} signals that reflect cellular physiology.

Changes in intracellular Ca^{2+} were assessed by imaging a fluorescent, C_{a}^{2+}-sensitive dye (flu-3 or flu-4). To image multicellular patterns of activity, it was necessary to use a

**D. Intracellular Ca^{2+} Transients in Neurons and Glia**

Brain mapping encompasses both structure and physiology. Mapping multicellular patterns of neural activity in brain tissue at high spatial and temporal resolution continues to be an important goal in neurobiology. One method that is proving useful is to image intracellular changes in calcium.
Figure 13 Three-dimensional organization of synaptic terminals in hippocampal brain slices revealed by synapsin-I (syn-I) immunostaining. Syn-I is a synaptic vesicle-associated protein found in virtually all CNS presynaptic terminals (DeCamilli et al., 1983a,b). Indirect immunohistochemistry with anti-syn-I antibodies and a fluorescein secondary antibody was used to label synaptic terminals in these fixed and permeabilized brain slices. The large fluorescent punctae correspond to mossy fiber giant synaptic terminals in hippocampal area CA3. (Top) Stereo-pair images of a syn-I-stained slice from a P13 rat. Giant synaptic terminals (arrows) occupy a dense band along the apical aspect of the pyramidal cell body layer. Small synaptic boutons (arrowhead), corresponding to non-mossy-fiber terminals, are seen within the cell body layer. Depth of view is 30 µm (15 images at 2-µm axial step intervals). (Bottom) Stereo-pair images from an organotypic brain slice prepared from a P7 rat and cultured for 14 days. Giant mossy fiber synapses (arrow) develop and maintain a normal distribution throughout the thickness of the cultured slices. Depth of view is 40 µm. Scale bar, 20 µm.

Figure 14 Use of antibodies to study the organization of postsynaptic structures in brain tissues. Hippocampal tissue slices (from P15 rat) were fixed and labeled with antibodies to PSD95, a prominent postsynaptic density scaffold protein at excitatory synapses. (A) Labeling in area CA3 reveals differences in staining patterns within the different strata. The stratum lucidum (SL) (between arrows), corresponding to the primary mossy fiber axon tract, contains many giant postsynaptic density (PSD) clusters. However, some giant clusters are also found along basal dendrites in the stratum pyramidale (SP). (B) Higher magnification view showing large postsynaptic clusters (arrowhead) in the SL. Only small synapses are evident in the stratum radiatum (SR). (C) In contrast to area CA3, no large synaptic clusters are apparent in area CA1. Scale bar, 50 µm for A and C, 20 µm for B.
bulk loading procedure (using the membrane-permeant, AM–ester forms of dyes described earlier) so that most or all of the cells in the tissue slice were labeled. This method provided suitable labeling of neurons and glia in live hippocampal slices prepared from developing rat. Time-lapse imaging showed that developing pyramidal neurons exhibit spontaneous Ca\(^{2+}\) transients that reflect neural synaptic activity (Dailey and Smith, 1994). Although most pyramidal neurons

**Figure 15** GFP labeling of synapses in transfected neurons in a hippocampal slice. Using a gene gun, neurons were transfected with a plasmid vector encoding a GFP–PSD95 fusion protein to label postsynaptic densities (PSDs). For details, see Qin et al. (2001). (A) Five neurons were transfected in this field of view from area CA3. (B) Higher magnification, stereo image of the cells above showing small postsynaptic structures on secondary dendrites in the SR and large postsynaptic structures on primary apical dendrites in the SL. The large PSD clusters (arrow) correspond to synaptic contacts of the mossy fibers. Use red–green stereo glasses (available from the author) to view depth in the image. (C) Higher magnification view of cells shown at lower right in A. Note the differences in morphology as well as patterns of GFP–PSD95 localization in these two adjacent neurons. The cell on the left (*) bears large GFP–PSD95 clusters (arrows) typical of mossy fiber synapses on a CA3 pyramidal neuron (cf. A and B). The cell on the right, which has a distinctive morphology with long, thick dendrites bearing few branches, is densely covered with small synapses but no large clusters. (D) Stereo image of cells shown in C. These images demonstrate the feasibility of mapping all morphological synaptic structures on individual neurons, as well as distinguishing neurons on the basis of synaptic organization.
appear to be independently active, occasionally there are small groups of synchronously-active neurons (Fig. 16). Such patterns of activity may reflect electrical coupling between neurons during development. The significance of these activity patterns remains unclear, although similar patterns of activity are found in the developing neocortex and are thought to help set up the organization of chemical synaptic contacts between groups of neurons (Yuste et al., 1992).

Figure 16 Patterns of spontaneous neural activity in brain slices from developing rat hippocampus. Neural activity is detected by a Ca²⁺-sensitive fluorescent dye, fluo-3. An increase in fluorescence intensity corresponds to a rise in intracellular Ca²⁺. Each image is a composite of three separate images taken at 12-s intervals and color coded such that different colored cells were active at different times. Both fields (A and B) show activity of pyramidal neurons in area CA1 of hippocampal slices from a P3 rat. Very few glial cells are evident at this developmental stage. (A) Note the high level of spontaneous Ca²⁺ activity in pyramidal cell bodies and apical dendrites. Activity of adjacent neurons is largely nonsynchronous. (B) Small groups of synchronously active neurons (red cells at arrow) are occasionally seen at this stage of development. This pattern of activity is likely due to the coupling of pyramidal neurons via gap junctions. Coupling between neurons is known to occur during development but diminishes as chemical synaptic connections are established. Scale bar, 25 µm for A, 50 µm for B.
A qualitatively different pattern of neuronal activity is found when hippocampal slices are maintained in culture for several weeks. When slow-scanning confocal images are collected, horizontal bands of high Ca²⁺ in neuropil and in neuronal cell bodies are seen (Fig. 17). Such patterns of neural activity probably reflect epileptiform-like activity, which is known from electrophysiological studies to develop in these cultured slices (McBain et al., 1989).

**Figure 17** Patterns of spontaneous epileptiform activity in hippocampal slices detected by slow-scanning confocal imaging. (A) Composite image showing Ca²⁺ activity at three different time points (encoded blue–green–red, respectively) collected at 7-s intervals. At two of the three time points, a burst of synchronized activity was evident in many neurons (green and red horizontal stripes). Such patterns of activity are indicative of synchronized bursting, which is known from electrophysiological studies to occur in these brain slice cultures. The horizontal patterns are due to the slow scan rate (about 10 ms per horizontal line of resolution in this case) relative to the rate of Ca²⁺ rise in the cells. Note the high level of fluorescence near the onset of activity (arrows) followed by a gradual decline in fluorescence intensity as the scan collects data from portions of the field below. Scale bar, 25 µm. (B) Graphical display of spontaneous synchronized Ca²⁺ activity in neurons. Traces of fluorescence intensity over time are shown for eight neurons that were situated roughly in a horizontal line across the image field so that the time of sampling was identical for all these neurons. Images were collected at 7-s intervals. Initially, there was a low level of spontaneous activity. At about the 9-min time point, the neurons started to exhibit synchronized Ca²⁺ activity (upward spikes) that persisted for many minutes.
When the cultured brain slices are imaged at a higher time resolution with a fast scanning confocal microscope, the time course of the fast neuronal Ca\(^{2+}\) transients associated with epileptiform activity is better resolved. A comparable pattern of activity can be pharmacologically induced in cultured brain slices perfused with GABA\(_A\)-receptor antagonists, picrotoxin (100 µM) or gabazine (13 µM), which disinhibit brain slices (Fig. 18). These images serve to demonstrate the trade-off between high spatial and temporal resolution in scanning confocal imaging (see earlier discussion). Collection of image data at high rates generally reduces the spatial resolution because many fewer photons are collected at each point in space.

In addition to neuronal activity, imaging fluo-3-labeled brain slices with the confocal microscope reveals patterns of glial cell activity (Figs. 18C and 19). Previous studies revealed that transcellular waves of activity can pass through gap junctions connecting astrocytes (Cornell-Bell et al., 1990). In brain slices, the intracellular glial Ca\(^{2+}\) activity has been linked to the local release of neurotransmitter during neural synaptic activity (Dani et al., 1992). These observations suggest a dynamic, functional interplay between the neuronal and the glial networks in brain tissues (Smith, 1994; Charles, 1998; Araque et al., 2001; Bezzi and Volterra, 2001).

**VI. Conclusions and Future Prospects**

The development of novel fluorescent probes of cellular structure and physiology (Mason, 1993; Tsien and Waggoner, 1995) has had a profound impact on studies of brain structure and function at the network, cellular, and subcellular levels. Coupled with the technical advances in high-resolution optical imaging, fluorescent markers provide a valuable set of

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**Figure 18** Patterns of picrotoxin-induced epileptiform activity in hippocampal slices detected by fast-scanning confocal imaging of intracellular calcium. The high time resolution was achieved using a confocal microscope (Noran Odyssey) that collects full-field images at video rate (30 Hz). (A) A single scan (nonaveraged) image showing the relatively low spatial resolution of fluo-3-labeled pyramidal neurons when imaged at high time resolution (compare with slow-scanned images in Figs. 16 and 17A). The slice is oriented such that the pyramidal cell body layer (SP) runs in a horizontal band across the middle of the field. Numbered boxes indicate the location of some of the pyramidal cell bodies, which were more clearly evident in averaged images. SR, stratum radiatum. SO, stratum oriens. Scale bar, 20 µm. (B) Plots of fluorescence intensity over time for the five boxed regions, corresponding to five pyramidal cell bodies, in A. Note how the high time resolution of the fast-scanning imaging reveals the repetitive, synchronized Ca\(^{2+}\) spikes in the neurons. (C) Plots of fluorescence intensity for a neuron (upper trace) and for four other nearby cells (not shown) that are most likely glia. Note the various patterns and slower time courses of Ca\(^{2+}\) transients in the nonneuronal cells that are resolved with the fast-scanning imaging.
tools for mapping the functional organization of the brain. The wide variety of fluorescent membrane dyes now available with varying spectral properties permits simultaneous labeling and discrimination of different populations of cells. This will continue to be useful to hodologists interested in more precisely identifying the organization and interrelation-ship of neural projections in brain tissues.

One area of great potential growth is in the development of new structural and functional probes that would be useful for identifying specific populations of neurons or for labeling functional synaptic contacts in live brain tissues. Ideal probes will permit functional analysis at high spatial and temporal resolution. The widespread use of GFP and similar proteins indicates that genetically engineered fluorescent probes should continue to play an important role in elucidating neural structure and function (see Yuste et al., 1999a). New probes that are likely to participate significantly in this endeavor include calcium-sensitive (Miyawaki et al., 1997), pH-sensitive (Miesenbock et al., 1998), and voltage-sensi-tive (Siegel and Isacoff, 1997, 1999; Sakai et al., 2001) fluorescent proteins, especially those that are targeted to synapses (Sankaranarayanan et al., 2000). It seems likely that genetic probes whose expression can be restricted to certain brain regions or cell types will contribute signifi-cantly toward mapping the functional anatomy of the brain (Spergel et al., 2001). Already, there are several transgenic mouse models that express reporters such as GFP in restricted subsets of neurons (van den Pol and Ghosh, 1998; Oliva et al., 2000) or glia (Zhuo et al., 1997).

Given the advancements in vital fluorescent probes and sensitive imaging techniques, it is now possible to map the 3D structure of single neurons and glial cells in live brain slices over a period of many hours. The ability to collect 2D and 3D image data sets from live neural tissue slices at high spatial resolution, over long periods of time and at relatively short time intervals, is revealing new information on the dynamics of neural structure in brain tissues. The time-resolved 3D imaging methods described here capture more of the dynamic events occurring within tissue and also provide the researcher with assurance that observed structural changes are not due to movement in and out of a focal plane. We can expect these vital fluorescence labeling and imaging methods to be applied more widely to studies of structural and functional neural organization in a variety of brain regions, especially with the use of genetic probes that can be targeted to specific brain regions and cell types. Moreover, it is anticipated that cell-type-specific probes will enable new strategies for investigating structural and functional relationships of neurons and glia.

Much can be done with the existing optical imaging technology. Nevertheless, future developments will undoubtedly continue to address constraints on high-resolution imaging deep (>50 µm) within tissues. Improvements are already being realized by using water-immersion lenses (to reduce spherical aberration) and longer wavelength dyes (to reduce...
light scatter by the tissue and minimize phototoxic effects). Newer optical techniques such as multiphoton imaging (Denk et al., 1990, 1994) are being used more widely and are making significant contributions to mapping neural structure and function. Multiphoton imaging provides intrinsic three-dimensional resolution (Williams et al., 1994) while confining fluorescence excitation to a single narrow focal plane, thus reducing the risk of photodynamic damage. With regard to imaging cellular and subcellular structure, multiphoton excitation yields a substantial improvement over conventional confocal for imaging fluorescently labeled cells at deeper (100-500 μm) levels in live tissues (Mainen et al., 1999; Majewksa et al., 2000).

Another very exciting optical technique that should greatly facilitate the mapping of functional neural organization in brain slices is based on laser photostimulation (Farber and Grinvald, 1983). A modification of this technique (Callaway and Katz, 1993; Katz and Dalva, 1994) employs a scanned laser beam to focally release a caged (photoactivatable) form of the excitatory neurotransmitter, glutamate, thus stimulating nearby neurons. The development of new probes that can be uncaged with multiphoton excitation (Augustine, 2001; Matsuzaki et al., 2001) should enable higher resolution studies in more intact tissues. In conjunction with electrophysiological recordings or physiological imaging, these methods should continue to provide opportunities for high-resolution mapping of neural circuits in excised brain slices.

At present, most of the work at the cellular and subcellular levels of resolution is being done in excised tissue slices. However, future work will continue to probe more of the outstanding questions in intact, functioning preparations (e.g., Dirnagl et al., 1991; Them, 1993; Svoboda et al., 1999; Lendvai et al., 2000; Ilyin et al., 2001). At any rate, it is clear that neuroscientists have a repertoire of powerful optical tools for dissecting and mapping the functional organization of brain tissues.

Acknowledgments

Some of the work described herein was performed by the author in the laboratory of Dr. Stephen J. Smith (Stanford University). More recent work in the author’s laboratory at the University of Iowa was supported by grants from the NIH (NS37159) and Whitehall Foundation (98-6).

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3 Optical Imaging of Neural Structure and Physiology


II Surface-Based Data Acquisition


