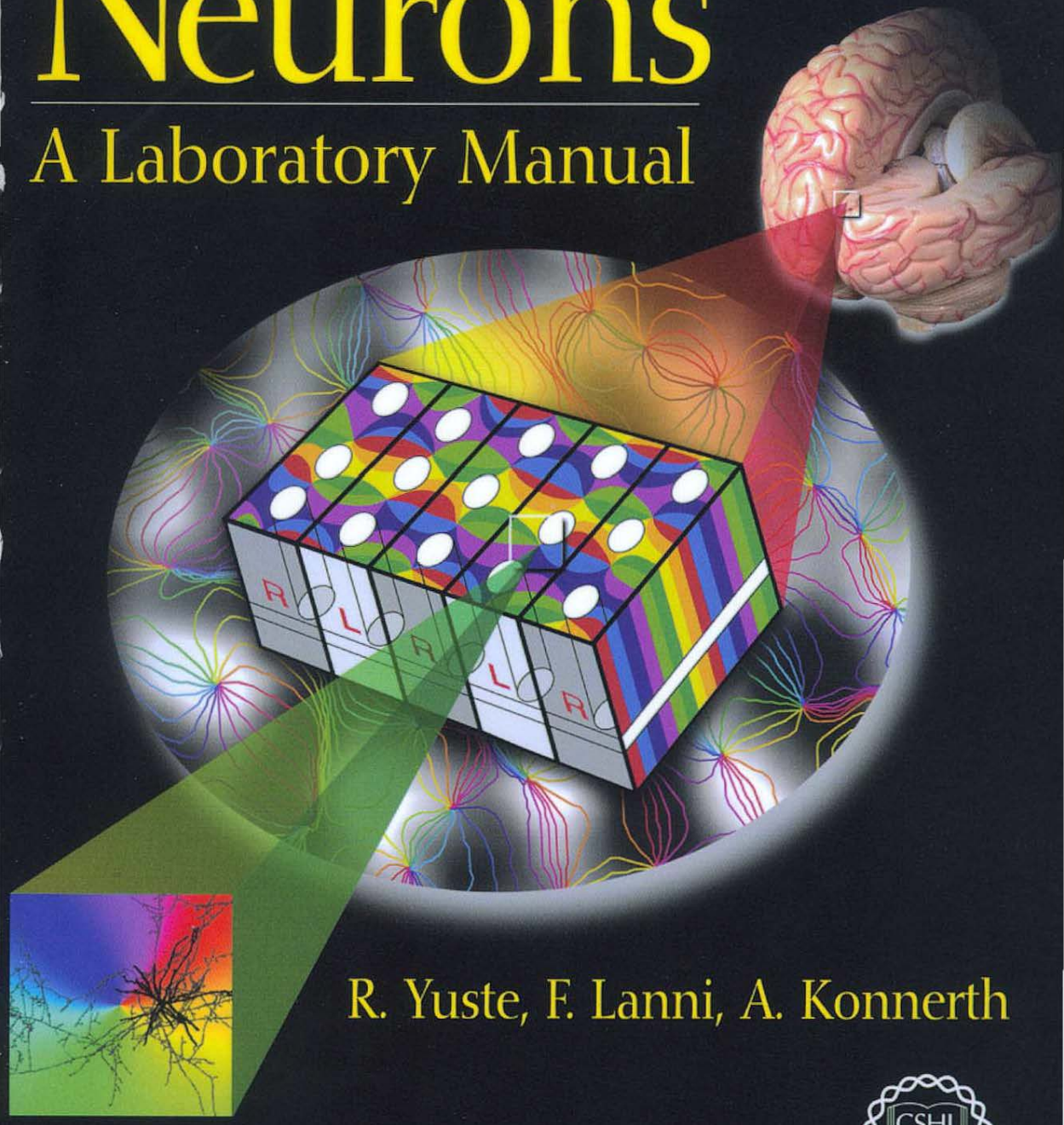
Imaging Neurons

A Laboratory Manual



R. Yuste, F. Lanni, A. Konnerth

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Two-photon Microscopy for 4D Imaging of Living Neurons

STEVE M. POTTER

INTRODUCTION

Two-photon laser scanning fluorescence microscopy (Denk et al. 1990) has made it possible to image neurons over 600 microns deep within a living slice or organism, with submicron resolution and in three dimensions (3D) for many hours without photodamage (Potter et al. 1996a). With true 4D microscopy (i.e., 3D with time), it is now feasible to capture neural development and synaptic plasticity in the *act of happening*, eliminating many uncertainties associated with between-animal comparisons of fixed-tissue specimens. By using pulsed IR laser light to excite fluorescent labels (e.g., dyes, fluorescent proteins, or endogenous fluorophores) that are normally excited by visible light, excitation is restricted to the focal plane, greatly reducing photobleaching and phototoxicity (see Chapter 17). The IR illumination is scattered less than visible light, allowing imaging 2–3 times deeper than with standard confocal microscopy. In addition, because the fluorescent signal emanates only from the focus of the scanning IR laser beam within the specimen, no confocal aperture is necessary to remove the out-of-focus signal (see Figure 22.1 in Chapter 22). This means that two-photon microscopy has an inherently higher S/N ratio compared with confocal microscopy. Excellent references for two-photon microscopy, and for labeling and imaging living specimens, include Denk et al. (1995) and Terasaki and Dailey (1995) (see also Chapter 10).

In this chapter, I describe our two-photon imaging hardware, pointing out potential pitfalls in microscope construction and operation. I also describe technical considerations for successful two-photon microscopy in two experimental systems: (1) 4D imaging of living neurons transplanted to cultured hippocampal slices from neonatal rats and (2) 4D imaging of dendritic spines within acute hippocampal slices from adult rats. Figure 20.1 shows the components of the imaging setup.

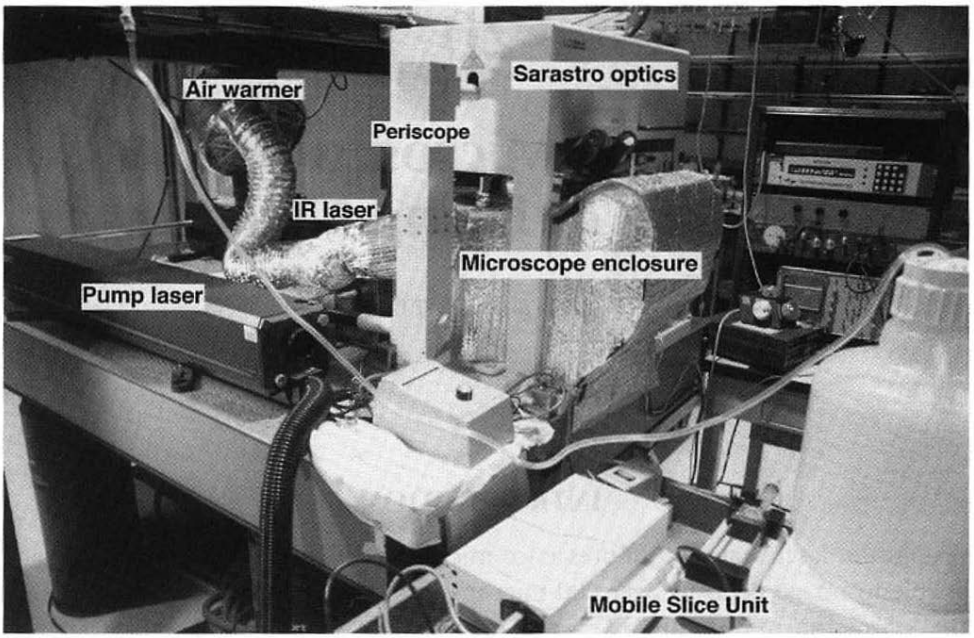

Figure 20.1. The imaging setup. In the foreground is the Mobile Slice Unit, with oxygenated ACSF tank and peristaltic and syringe pumps. In the center is the Molecular Dynamics Sarastro 2000 confocal microscope (converted for two-photon microscopy), enclosed in insulation and warmed by an egg incubator (*upper left*). Behind the microscope are the argon-ion pump laser and Ti:sapphire IR laser. The argon-ion laser has been replaced with a shoe box-sized, all solid-state Verdi pump laser (not shown).

TWO-PHOTON HARDWARE

Although both confocal and two-photon microscopes scan a laser beam in the focal plane within the specimen, a two-photon microscope can actually be a much simpler device than a confocal microscope. There is no need to focus or even descan the emitted fluorescence to create an image. In most cases, however, it is probably easier to convert a confocal microscope to a two-photon microscope than to build a two-photon system from the ground up. I converted a Molecular Dynamics Sarastro 2000 confocal with an upright Nikon Optiphot II microscope, preserving its ability to be used in standard confocal mode (Potter et al. 1996c) (see Figure 20.2). In every case where Professor Scott Fraser and I imaged the same specimen using confocal microscopy with visible excitation (either the Sarastro or the Bio-Rad 600) and two-photon microscopy (converted Sarastro), we got better resolution, deeper penetration, and less photodamage with two-photon microscopy. The confocal capabilities of the converted Sarastro are therefore seldom used.

Lasers

To produce adequate two-photon excitation, an IR laser that compresses all its output into very short (~100 fsec) pulses is necessary. Although the peak power of these puls-

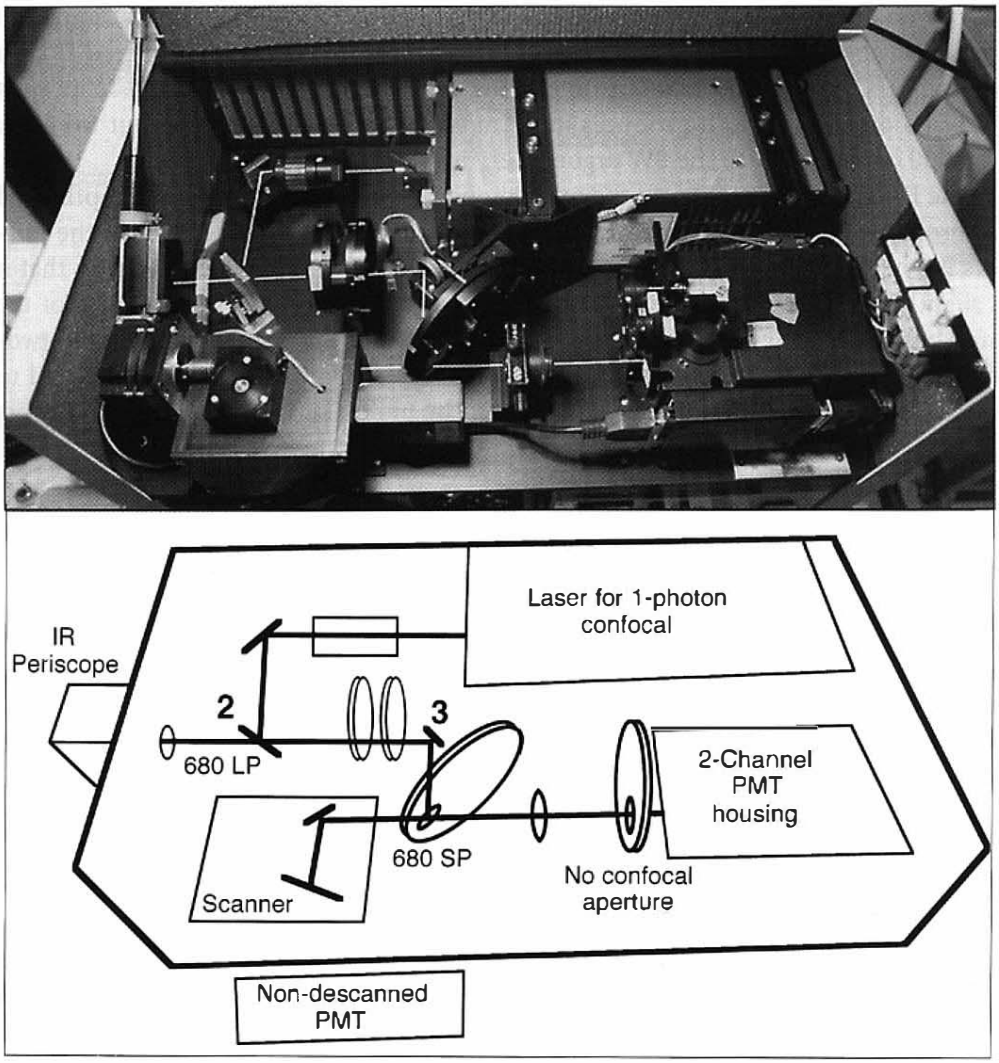

Figure 20.2. Optics of Molecular Dynamics Sarastro 2000, modified for two-photon microscopy. The pulsed IR laser beam is brought into the Sarastro by a periscope to the left. Before the periscope is a 3x "beam expander" (not shown) used to match the IR laser beam width to the back aperture of the objective lens. To switch from two-photon to confocal imaging, one need only block the IR beam, turn on the on-board laser (*upper right*), rotate the primary dichroic beamsplitter wheel to a long-pass mirror, and rotate the pinhole aperture wheel from the open hole to one of the three pinhole settings.

es is enormous, the pulses are widely spaced, so the mean power is not enough to cause any heating of the specimen. (For a detailed discussion on lasers in multiphoton microscopy, see Chapter 18.)

We use the Coherent Mira900 titanium:sapphire laser, which is tunable from approximately 700 to 1000 nm. The Ti:sapphire laser converts green light from a pump laser into pulsed IR light.

The Coherent Verdi has replaced our previous pump laser, a Coherent Innova310 8W argon-ion laser. The Verdi is an all-solid-state laser that can be powered from a normal 110 V outlet and requires no cooling water, unlike the Innova310. With only 5.5 watts of green light, it produces more mode-locked (pulsed) IR output from the Mira900 compared with the 8W argon laser, due to improved beam quality.

The IR beam is brought into the microscope with four dichroic mirrors, optimized for broadband IR reflection at a 45° angle (Newport BD.2). Between two of the mirrors is a beam expander (Newport T81-3x), which is used to focus the beam so that it is slightly larger than the back aperture of the objective lens. Without it, most of the large beam from the trinocular (scanner) eyepiece does not enter the lens, and two-photon excitation is greatly reduced. All beams are covered with tubes connected to sealed mirror boxes. This allows safe use even by biologists, and protects the mirrors from dust buildup and accidental bumping.

Conversion of the Sarastro 2000 Confocal to a Two-photon System

The Sarastro 2000 has a very simple light path compared with most available confocals, which is desirable because valuable photons are inevitably lost at every mirror, lens, and filter. To convert it to a two-photon microscope, I replaced mirror 2 (see Figure 20.2) with a 680-nm long-pass (680 LP) beamsplitter that allows us to use either the IR laser or the on-board visible laser. We use a 680-nm short-pass (680 SP) dichroic mirror to separate excitation from emission in the primary beamsplitter wheel. Mirror 3 and the two scanning mirrors were coated with silver and a protective dielectric coating (Ventura Optical Industries). Silver reflects both visible and IR light well, unlike standard aluminum mirrors, which reflect IR poorly. The dielectric is crucial to prevent the silver from tarnishing. No changes were made to the OptiphotII, which has a motorized z-stage with 0.1-μm accuracy. The stage and scanner are controlled by an SGI Indigo workstation, running Molecular Dynamics ImageSpace software. No changes to the software were necessary for two-photon imaging.

Non-descanned Detection: Every Photon Is Sacred

To get images with the best S/N ratio, it is important to collect as many photons as possible. Our microscope sends the emitted light through a trinocular eyepiece (beneath the scanner), two scanning mirrors, and an achromat lens before it gets to the on-board detectors. To avoid the losses associated with these optics, and to collect more of the photons that are scattered on their way out of the specimen, it is best to detect the emitted light as close to the specimen as possible (see Figure 20.3). We replaced one of the OptiphotII's epifluorescence filter cubes with a custom cube (Omega Optical) that transmits IR from above and reflects visible fluorescence forward to a photomultiplier tube (PMT; Hamamatsu R928) that we installed just below the binocular eyepiece.

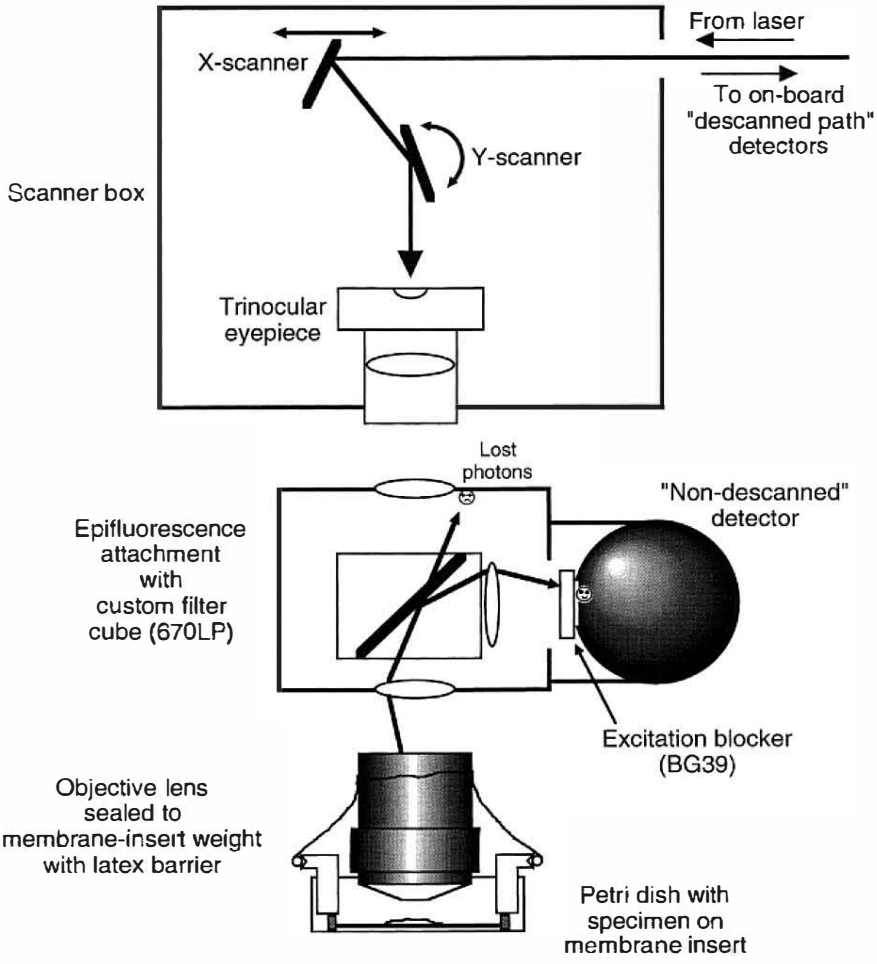

Figure 20.3. Non-descanned detection. By placing a detector close to the specimen, it is possible to collect more scattered photons and avoid losses associated with optics in the descanned path. For low background, this "non-descanned" detector must be well shielded from room lights and scattered IR excitation. To avoid damage to the sensitive photomultiplier tube, it should be automatically shut off when the transilluminator, the epi-illuminator, or the on-board laser is turned on. The Y-scanner reflects the IR beam down through a trinocular eyepiece and custom filter cube to the objective lens. Emitted fluorescence is reflected by the cube into a custom detector mounted directly in front of it, or if the cube is pulled out, it is descanned and sent through a 680-nm short-pass beamsplitter and achromat lens to the on-board dual-channel detector (descanned path).

One antireflection-coated lens in the filter cube allows scattered photons that would not have made it to the on-board ("descanned path") detectors to enter this "non-descanned path" detector.

It is crucial to protect the PMT from scattered and reflected excitation light by sealing a BG39 colored-glass filter to the PMT housing. (A different filter may be more appropriate for red-emitting fluorophores.) I installed circuitry to turn off the PMT

when any of the microscope's other light sources are turned on (substage tungsten lamp, epifluorescence lamp, or on-board confocal laser), to prevent accidental damage to the PMT. Although the original detectors are much less sensitive than the non-descanned detector, they are still used for double-labeling experiments.

IMAGING NEURONS TRANSPLANTED TO CULTURED SLICES

Survival of transplanted neurons is often disappointingly low (Shetty and Turner 1995). Professor Jerry Pine and I developed a model system for neuronal transplants that allows continuous imaging of transplant integration into the host tissue (Potter et al. 1996b; Fraser et al. 1997), to shed light on the dynamics of transplant migration and integration success or failure. The idea is to label a suspension of neurons from embryonic rat hippocampus with fluorescent membrane dye, wash away all free dye and labeled debris, and seed the cells onto cultured hippocampal slices from neonatal rats (see Figure 20.4). 4D two-photon imaging showed that after one day in culture, the transplanted cells migrated throughout the slice and began to extend axons and dendrites. For an example of such a 4D movie, see the following WWW site:

http://www.caltech.edu/~pinelab/movies.html

At 17 days, transplanted neurons had developed pyramidal morphologies, with processes extending for several hundreds of microns (Potter et al. 1996b).

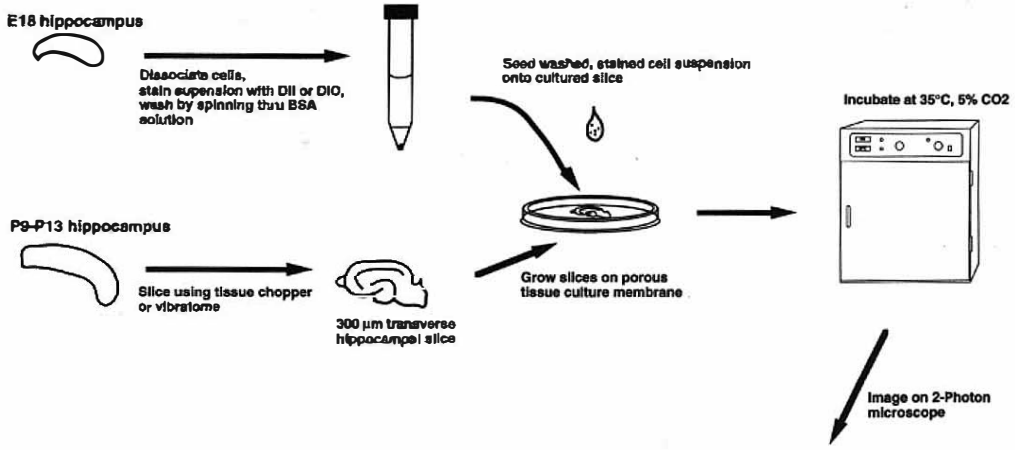

Figure 20.4. "In Slico" transplant model. Cells from an embryonic rat hippocampus are labeled and seeded onto cultured neonatal hippocampal slices. After some time in culture, transplant integration is observed in living slices using two-photon microscopy.

Cell Labeling

Lipophilic dyes, such as DiI or DiO (generic acronyms for dialkylindocarbocyanines and dialkyloxacarbocyanines, respectively), are often used to trace living neuronal processes. Small structures such as axons, filipodia, and dendritic spines contain little cytosol and are better visualized with a membrane dye than a cytosolic dye. The problem is that these dyes tend to crystallize in aqueous media. Dye crystals are difficult or impossible to remove from a cell suspension, and will label host neurons if transplanted with the labeled cells. To surmount this problem, I used the more soluble 12-carbon form of DiI, along with a nonionic macromolecular surfactant Pluronic F127 (Lojewska and Loew 1986, 1987), to ensure that the dye remains completely dissolved and to aid the transfer of dye to the cell membrane. I have also used the 16-carbon form of DiO, which is slightly less soluble. Normally excited by blue light (485 nm), DiO is easier to excite with the Ti:sapphire laser than DiI (normally excited by green light around 548 nm). The following methods are from Potter et al. (1996b), which has further details.

Preparation of Clean Cell Suspensions

1. Cut brain tissue into 1-mm pieces. Digest with 0.25% trypsin, 0.02 mg/ml DNase (Sigma) in Hanks' balanced salt solution (no calcium or magnesium [Gibco/BRL]) at 37°C for 15 minutes.
2. Gently wash pieces in plating medium. Repeat wash.

   Note: For hippocampus, we use Neurobasal with B27 supplement (Gibco/BRL) and 500 µM glutamine, 25 µM glutamate.

3. Gently triturate in 1 ml of plating medium with five passes through the 0.78-mm opening of a blue tip of a P-1000 Pipetman.

   Note: This is much more reproducible than with the ubiquitous "fire-polished pasteur pipet."

4. Decant suspended cells and triturate the remaining pieces once more (see step 3, above).
5. Gravity-filter cell suspension through a 70-µm nylon mesh (Falcon) to remove large debris.
6. To remove small debris, layer 0.5 ml of 5% bovine serum albumin in Ix phosphate-buffered saline (BSA/PBS) under cell suspension in a 15-ml tube. Centrifuge the cell suspension through the BSA/PBS layer in a swinging-bucket rotor at 160*g* for 2 minutes.
7. Discard supernatant and resuspend cell pellet in plating medium by gentle trituration (three passes) as described in step 3 (above).

DiI Labeling of Neurons in Suspension

1. Warm staining solution to 37°C in a water bath. Add approximately 0.5 ml of cell suspension (from step 7, above) with gentle mixing to the staining solution.

Staining Solution

Prepare a 40 mg/ml stock solution of $DiIC_{12}$ (Molecular Probes) by dissolving the tar-like dye in **DMF** containing 2.5% (w/v) **Pluronic F127** (BASF). This solution can be stored at –20°C and dissolved easily upon warming to room temperature.

To prepare staining solution, add 2 µl of dye stock to 2 ml of plating medium in a 15-ml tube (final concentration: 40 µg/ml $DiIC_{12}$, 0.0025% Pluronic F127, 0.1% DMF). This solution should easily pass through a 0.2-µm sterile filter with no diminution in color, indicating that it is completely dissolved.

Note: Labeling will be incomplete at room temperature due to decreased membrane turnover.

DMF, Pluronic F127 (see Appendix 4 for Caution)

2. Incubate cells at 37°C for 15 minutes.
3. Layer 0.5 ml of 5% BSA/PBS under the cells in staining solution using a long pasteur pipet.
4. Centrifuge in a swinging-bucket rotor at 160*g* for 6 minutes. Carefully decant the staining solution floating above the BSA/PBS, as well as most of the BSA/PBS.
5. To resuspend the stained cells, triturate (three passes) in 1 ml of plating medium using the P-1000 as described in step 3 (p. 20.7).
6. Repeat steps 3–5 (above) to remove any remaining dissolved dye.

Labeled Transplants to Slice Cultures

These static (membrane) cultures are prepared according to the method developed by Stoppini and coworkers (Stoppini et al. 1991; Buchs et al. 1993). Routinely grown over 1 month in culture, they are several cell layers thick (~150 µm) and retain more of the original architechtonics than do roller-tube cultures.

1. Cut hippocampal (or other brain tissue) slices (300 µm) from postnatal day 7–11 rat pups using a tissue chopper or vibratome, keeping the slices submerged in Hanks' solution.

2. Add 1 ml of Organotypic Slice Culture Medium (Vanderklish et al. 1992) to a 35-mm petri dish and place a Millicell-CM membrane insert upon the medium.

Organotypic Slice Culture Medium

minimal essential medium (MEM) with Hanks' salts, no glutamine (GIBCO-BRL)
25% horse serum (Hyclone)
30 mM glucose
5 mM $NaHCO_3$
30 mM HEPES
2.5 mM **$MgSO_4$**
2 mM **$CaCl_2$**
3 mM L-glutamine
1 mg/liter insulin (Sigma)
10 ml/liter penicillin/streptomycin (Sigma P0781)

Adjust pH to 7.2 at 35°C.

Notes: The membrane becomes clear when wet, facilitating imaging from below.

The plastic ring on standard inserts gets in the way of petri dish lids, electrodes, and large objective lenses (upright scope), so I used to cut it off with a hot nichrome wire. I convinced Millipore to produce "Low height" inserts (#PICM0RG50) to avoid this hassle.

$CaCl_2$, $MgSO_4$ (see Appendix 4 for Caution)

3. Transfer 1–3 slices to the membrane using a small spatula whose edges have been rounded with a grindstone.

Note: The culture medium soaks through the membrane from below, and the moist slice is well oxygenated from above.

4. Incubate slices at 35°C in humidified 5% carbon dioxide atmosphere. Replace half the medium under the membrane with fresh medium weekly.

Note: Slices will adhere well to the membranes in a day or two and flatten out to about 150 µm. For submerged imaging sooner than this, the slices must be glued to the membrane using a clot of fibrin (Brown et al. 1993).

5. Dilute freshly labeled cells (from step 6, p. 20.8) to 50,000 cells/ml in slice culture medium.

6. Place a drop (~5 µl) of cell suspension on each slice, at least one day after preparing slice cultures.

Notes: In a few minutes after addition of the cell suspension, excess medium will soak through the membrane, and the cells will settle on and adhere to the slice. I have found that within a day, embryonic hippocampal neurons have migrated throughout the 150-µm thickness of a cultured slice.

This method provides 20–50 labeled cells per slice.

Environmental Control during Imaging

I enclosed the microscope body within Mylar bubble-plastic insulation (see Figure 20.1), connected to a chicken egg incubator (Marsh Automatic Incubator, Lyon Electric Co.) to warm the air to rat body temperature. Using this low-wattage heater (100 W), there are no thermostat-related temperature fluctuations. It takes about 12 hours for the microscope stage, objective, and other components of the setup to warm up to 35°C, but everything remains thermally stable during the imaging. This means there is no focus drift during protracted imaging sessions, even if the room temperature changes. The opaque enclosure also serves to block ambient light from entering the objective and causing unwanted background signal. This is especially important when using the sensitive non-descanned detector. For slices that have adhered to the membranes, I usually flood the slice with slice culture medium during imaging, and use a 40x/0.75-NA Nikon or 63x/1.2-NA Zeiss water-immersion objective. For nonadhered slices, it is necessary to glue slices to the membrane using a fibrin clot (Brown et al. 1993). The membrane insert is weighted down by a custom-made, stainless steel ring. To prevent evaporation of medium and pH drift, a condom with the tip cut off (Trojan nonlubricated, Carter-Wallace, Inc.) seals the ring to the objective lens (see Figure 20.3). Because the objective lens is warm, it is also possible to image a moist nonflooded slice using an air objective, without condensation fogging the lens.

Data Acquisition

The two-photon absorption maximum for DiI is approximately 1030 nm (Xu et al. 1996), and the power of the Ti:sapphire laser falls off from 900 to 1000 nm; thus, an empirically determined excitation wavelength of 960 nm seems to give the best images of DiI-labeled specimens. At 900 nm, where the laser is more powerful, background autofluorescence increases more than DiI fluorescence. For imaging neurons labeled with DiO, two-photon excitation anywhere from 850 to 900 nm produces excellent signal-to-background ratios. Although photodamage is tens or hundreds of times less with two-photon microscopy than with standard wide-field fluorescence or confocal microscopy, it is possible to use too much laser power, excite already-excited fluorophores, and cause excessive dye bleaching. For this reason, I always set the PMT at 80–100% of its maximum voltage, and reduce the laser power to the minimum necessary to provide a good fluorescence signal at this gain, usually 10–30% transmission.

Each time point of a 4D time-lapse movie consists of a series of 20–50 *z*-sections, taken at 0.3- to 2-µm increments. With a 40x objective, a volume as large as 250 x 250

x 100 µm can be imaged every 5 minutes for over 8 hours. As long as the above guidelines are adhered to, there is no sign of photobleaching or phototoxicity of labeled neurons. To save on data storage and post-processing time, one can collect widely spaced (2 µm) *z*-sections and average two or three scans per section to reduce shot noise. However, better noise reduction is obtained by taking more single-scanned sections that are more closely spaced (0.5 µm or less), and then subjecting them to a 3 x 3 x 3 median filter using ImageSpace (or equivalent) software. Using 3D filters is only valid when each section is very similar to the two adjacent sections.

TWO-PHOTON, 4D TIME-LAPSE IMAGING OF DENDRITIC SPINES

In addition to its usefulness for revealing the dynamics of developing neural systems, two-photon microscopy is useful for revealing potential morphological correlates of synaptic plasticity in adult brain tissue. In collaboration with Professor Erin Schuman and David Kantor, I have imaged dendritic spines in acute hippocampal slices from juvenile rats, to study structural changes associated with synaptic facilitation.

Maintenance of Healthy Slices

Maintaining acute slices poses a number of technical difficulties compared with organotypic cultured slices. The adult hippocampus is much more susceptible to excitotoxic injury from anoxia than the neonatal hippocampus. Adam Mamelak, M.D., and I developed the Mobile Slice Unit (see Figure 20.1), an instrument designed to keep acute slices healthy for up to 24 hours during imaging. It is a portable heart-lung machine that bubbles blood gas (95% oxygen/5% carbon dioxide) through artificial cerebrospinal fluid (ACSF) that is perfused continuously across the slice (150 ml/hour) by a peristaltic pump.

Medium Preparation

1. Equilibrate ACSF with blood gas (95% oxygen/5% carbon dioxide) several hours before use.

ACSF

25 mM glucose (Edwards 1995)
126 mM NaCl
2.5 mM **KCl**
1 mM **$MgCl_2$**
2 mM **$CaCl_2$**
1.25 mM **sodium phosphate**
26 mM $NaHCO_3$

$CaCl_2$, KCl, $MgCl_2$, sodium phosphate (see Appendix 4 for Caution)

2. Mix B27 nutrient supplement (Gibco/BRL) with ACSF using a syringe pump at 1:50 volume ratio.

   Note: B27 nutrient supplement is mixed with ACSF after bubbling of blood gas, to prevent foaming.

3. Carefully adjust both the pH and osmolarity to 7.3 and 295 mOsm, respectively.

   Note: Phenol red pH indicator is routinely included in the ACSF to ensure that the gassing is effective, because this is a carbonate-buffered solution. Ideally, an in-line pH sensor and oximeter would be included.

Temperature Control

As with cultured slices, we control the temperature of the microscope to prevent thermal focus drift, but usually conduct imaging at 25°C, because acute slices kept at 37°C survive for only 6 hours or so.

Measurements

We routinely check the field potential of slices before and after imaging and can induce long-term potentiation by tetanic stimulation after 20 hours of imaging, as long as the osmolarity, pH, oxygenation, and temperature are carefully controlled.

Slice Movement during Imaging

Acute slices are not adhered to a membrane as the cultured slices are. To prevent movement during imaging, I use slice weights that are a modification of those described in Edwards et al. (1989). A 1-mm-diameter silver wire is shaped into a "U" and pressed in a vise until it is 50 μm thinner than the acute slice, which is usually cut at 500 μm. This weight is then glued to a Millicell-CM insert using cyanoacrylate glue. The weight is cut out of the insert, leaving membrane in the middle of the U, and a small hole is cut in the membrane to allow access to the slice by electrodes or micropipets. The porous membrane slice weights are transparent when wet and do not cut into the slice as the nylon fibers of the weights used by Edwards et al. (1989) do.

To allow us to image several different sites during one time-lapse session, we attach the slice chamber to a Sutter MP-285 micromanipulator. The manipulator controller can remember the location of several interesting regions and return to them in sequence under computer control. As long as the slice is well weighted down, the positional accuracy is good to approximately 1 μm. This device also makes hunting around the slice for labeled cells much easier than using the microscope's mechanical stage translation knob, especially when imaging at high magnification.

Acute-slice Labeling

A number of approaches to labeling slices have been tried at Caltech and elsewhere. They include the following:

Biolistics and GFP

McAllister et al. (1995) have had great success using biolistics to shoot gold particles coated with GFP-encoding DNA into slices of developing ferret cortex. However, it takes 12 to 24 hours for GFP to be expressed and oxidized, which is not acceptable for acute hippocampal slices.

"Rusty-nail" Approach

We and other workers (Sorra and Harris 1997) have labeled slices by drying a solution of DiI or DiO on a glass pipet to form small crystals and poking the slice with the pipet to deposit the crystals within it. Although quicker than the biolistics approach, this also takes several hours for maximal labeling of dendritic arbors, presumably due to the small contact between dye crystals and cell membrane.

Oil-drop Approach

Hosokawa et al. (1992) and Dailey (Dailey and Smith 1993) have successfully labeled acute and cultured slices by dissolving DiI or DiO in fish oil and applying a small drop of this solution to the surface of the slice. Unfortunately, the cells best labeled by this technique are the unhealthy ones near the cut surface. Also, the oil drop must be removed by microaspiration before imaging, because it acts like a lens to defocus the excitation beam, reducing two-photon excitation.

Intracellular-dye Injection

We have produced some nice two-photon images of single neurons filled with fluorescein-dextran by intracellular iontophoretic injection. However, seldom do neurons survive for more than an hour after the trauma of impalement and electrode removal. Also, as mentioned on p. 20.7, membrane dyes produce clearer images of small structures such as dendritic spines.

Picospritzing a DMF Solution

We have had great success using a solution of DiO (16 mg/ml) in DMF. A very small amount can be applied by extracellular pressure injection into the middle of the slice. By varying pressure and pulse duration, any number from two to many neurons can

be labeled. Because the carrier is miscible with water, the dye surrounds the cells and they become maximally labeled within 30 minutes. We have not observed any toxicity from either the carrier or the dye.

Imaging and Post-processing

The demands and parameters for imaging spines in acute slices are similar to those used on cultured slices. However, to observe submicron changes in the structure of spines, which are typically 1–3 μm long and less than 1 μm wide, we use the smallest pixel size available. Using a 63x/1.2-NA water-immersion objective lens, this is 0.08 μm. Thus, a 512 x 512 scan is only 40 μm wide. This exacerbates any drift problems and makes control of temperature and osmolarity (which affects swelling or shrinking of tissue) even more critical. Inevitably, some drift or movement must be removed during post-processing, for example, by using the "register" function in NIH Image. This Macintosh software is available free at the following WWW site:

http://rsb.info.nih.gov/nih-image

To virtually eliminate noise and greatly increase the *z*-resolution of the images, we have found it helpful to perform image deconvolution of the raw data (see Figure 20.5). A model of how the microscope optics blurs the image is created by imaging subresolution fluorescent spheres (Molecular Probes MultiSpeck kit, M-7901) under

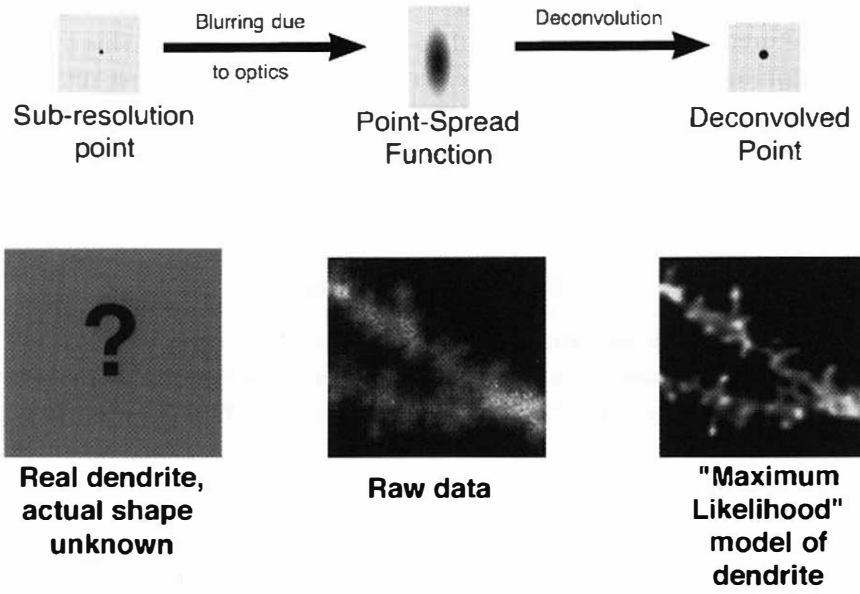

Figure 20.5. Image deconvolution. A 0.1-μm fluorescent sphere is imaged to obtain the microscope's point-spread function, which is then used to numerically deblur (deconvolve) a raw set of 3D-image data. We use the maximum likelihood estimation algorithm of the Huygens software suite.

conditions as similar as possible to the imaging of the dendrites. The Huygens System is presently the only deconvolution software that deals appropriately with multiphoton images (Vankempen et al. 1997). It takes several hours on an SGI O2 workstation (Silicon Graphics) to deconvolve a single *z*-series, but the improvement is dramatic. As with the 3D median filter, slices must be collected at very close intervals (0.5 μm or less) for effective deconvolution.

IMPROVEMENTS FOR THE FUTURE

The benefits of two-photon microscopy for imaging living specimens are clear. However, the technology will not be widely implemented until the lasers needed become much cheaper. Many labs that are not mechanically or optically inclined are waiting for a true "turn-key" two-photon system, one that is designed from the ground up for optimum IR throughput and efficient, non-descanned detection.

Although most labels that are in current use with standard fluorescence microscopy also work with multiphoton microscopy, the potential exists for dyes optimized for multiphoton imaging. In collaboration with Seth Marder at Caltech, we are developing new dyes with very large two-photon cross sections that promise greatly enhanced S/N ratios.

Fluorescent proteins provide relatively noninvasive labeling for a variety of specimens. In collaborations with Paul Garrity (Potter et al. 1996c) and Peter Mombaerts (Mombaerts et al. 1996), I have had great success using two-photon microscopy to image GFP-labeled neurons in living fruitfly optic lobes and mouse olfactory bulb, respectively. Transgenic mice expressing membrane-associated GFP in neuronal subpopulations would eliminate the need for tedious labeling procedures.

ACKNOWLEDGMENTS

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