

# Confocal Imaging of Microglial Cell Dynamics in Hippocampal Slice Cultures

Michael E. Dailey<sup>1</sup> and Marc Waite

Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242–1324

Methods are described for imaging the cellular dynamics of microglia in live mammalian brain slice cultures. Brain slices prepared from developing rat hippocampus are cultured for up to 2 weeks by the roller tube or static filter culture technique, stained with one or more fluorescent dyes, and imaged by scanning laser confocal microscopy. One of several cell type-specific or nonspecific fluorescent dyes can be used independently or in combination to label cells in live brain tissues. The fluorescently conjugated plant isolectin GSA-IB<sub>4</sub> is useful for identifying microglia and for following their structure, movement, and proliferation. Live and dead neurons and glia can be distinguished using membrane-permeant and -impermeant fluorescent nucleic acid dyes. Nonspecific fluorescent lipids such as DiIC<sub>18</sub> can be used as a vital stain to label populations of endocytic and phagocytic cells. Using multichannel confocal imaging, tissue slices that are single-, double-, or triple-labeled can be imaged in the living state in two or three spatial dimensions as well as in time. This provides a means for investigating the cell–cell interaction and dynamic behavior of microglia and other cell types in live brain tissues cultured under various physiological conditions. © 1999 Academic Press

Dynamic changes in neuronal and glial structure and organization play important roles in central nervous system (CNS) development, in normal physiological plasticity in the mature brain, and in the response of CNS tissue to injury. Understanding the nature and extent of changes in cell structure and tissue organization under various physiological conditions is an essential step toward elucidating the cellular substrates of CNS development, function, and repair. There is growing interest in understanding how cell structure and function changes in real time in the context of a

three-dimensional brain tissue environment. One exciting area of investigation is in the development of light microscope imaging methods for visualizing dynamic changes in cell movement, morphology, and physiology in real time within a native brain tissue environment (1).

Here we describe the use of hippocampal slice cultures as a model system for investigating the dynamics of cells, specifically microglia, in a three-dimensional brain tissue environment. Microglia play important roles in immunity and tissue reorganization in response to a host of neural tissue insults (2). Recent studies of isolated microglia in dissociated cell culture have begun to reveal the highly dynamic nature of microglial motility (3, 4), although their behavior *in situ* is much less clear. Nevertheless, the rapid mobilization of microglia during tissue remodeling makes them ideal candidates for studying the dynamics of motile cells in a complex, physiological brain tissue environment.

Probably the first direct time-lapse observation of microglial dynamics in live brain tissue was made by Smith and colleagues (5). These investigators soaked developing rat neocortical and hippocampal tissue slices in a fluorescent lipid, DiIC<sub>18</sub>, and using time-lapse confocal imaging observed a population of highly dynamic, phagocytic cells. These amoeboid cells were visible only by virtue of the “clusters of fluorescent cytoplasmic granules” that likely corresponded to internalized plasma membrane and/or phagocytosed debris derived from other fluorescently labeled cells. These macrophage-like cells were found to move rapidly throughout the tissue, often appearing to jostle or tug on neighboring cells along the way. More recent studies are confirming the identity of the amoeboid cells as microglia (5a), and we describe the development of methods for more selective labeling and imaging of microglia in living tissue. We also discuss the use

<sup>1</sup> To whom correspondence should be addressed. Fax: (319) 335–1069. E-mail: michael-e-dailey@uiowa.edu.

of vital fluorescent nucleic acid dyes that may be useful for distinguishing live and dead cells in CNS tissues. Together, these techniques should be useful for investigating the potential role of microglia in neuronal death (6) and in responding to neural tissue injury.

## DESCRIPTION OF METHOD

### A. Brain Slice Preparation

To assess the three-dimensional (3-D) morphology, organization, and dynamics of cells, brain tissue slices are prepared and examined immediately after labeling or after a variable period in culture. Tissue slices, whether examined acutely or after prolonged culture periods, can be imaged either in the living state or after chemical fixation.

#### 1. Acute Tissue Slices

For preparation of tissue slices from early postnatal (P3–P8) rodents, animals are swiftly decapitated and the heads are placed in ice-cold dissection medium, such as Hanks' balanced salt solution (HBSS; Gibco) supplemented with dextrose (6 mg/ml). Tissues of interest are dissected out and sliced at a thickness of 300–400  $\mu\text{m}$  using a McIlwain or manual tissue slicer (Stoelting Co., Wood Dale, IL).

#### 2. Cultured Tissue Slices

Two general approaches are currently being used for long-term culture of brain tissue (Fig. 1). These are referred to as "roller tube cultures" (7) and membrane or "filter cultures" (8). Both techniques produce so-called organotypic cultures that preserve much of the native tissue organization (9). Although these approaches have been used to culture tissues from a variety of brain regions, hippocampal tissue is perhaps most commonly used.

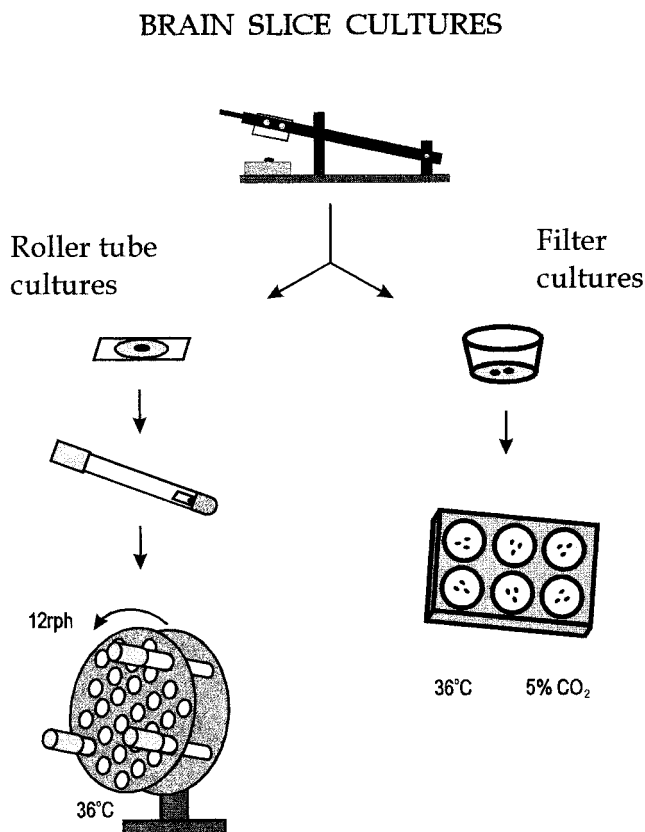
### B. Culturing

#### 1. Roller Tube Cultures

Techniques for the preparation and maintenance of roller tube cultures have been described elsewhere (7). The method involves securing a tissue slice to a rectangular (11  $\times$  22-mm) coverslip (Thomas Scientific, No. 6663-F10). Thorough cleaning of the coverslip is essential to promote attachment of the tissue. Coverslips are cleaned through a series of solvents—xylene (4 h), acetone (8 h), and ethanol (8 h)—on a rotator ( $\sim$ 100 rpm). Subsequently, the coverslips can be stored in 100% ethanol. Prior to use, coverslips are sterilized by flaming and placed in a large plastic Petri dish. We position 20 coverslips in a 150-mm Petri dish.

A plasma/thrombin clot has been the traditional method of choice for securing the tissue to coverslip. A

solution of chicken plasma is prepared from the lyophilized powder (available in 2- or 5-ml aliquots from Cocalico Biologicals, Reamstown, PA) which is reconstituted with distilled water. The plasma solution is prepared just before use and is filter sterilized using a 0.2- $\mu\text{m}$  syringe-tip filter. Bovine thrombin solution is made up at 100 U/ml in HBSS. The thrombin can be purchased in 1000-U vials (Sigma, St. Louis, MO), and the final working solution can be stored as frozen aliquots for up to a month. After thawing, the thrombin solution is filtered before use. To attach tissue slices, the tissue is transferred with a pipet to the coverslip, and excess buffer (HBSS) is removed. A small drop (10–15  $\mu\text{l}$ ) of plasma solution is added, followed by an equal volume (10–15  $\mu\text{l}$ ) of thrombin solution. It is important to remove excess buffer first so that the plasma and thrombin are not diluted significantly.



**FIG. 1.** Schematic illustration of major steps involved in preparation of organotypic brain slice cultures by the "roller tube culture" or "filter culture" technique. Tissue is dissected free, sliced with a tissue chopper, and either attached to pretreated glass coverslips (left) or placed on a filter insert membrane (right). In the case of the roller tube cultures, tissue is attached to coverslips using a plasma/thrombin clot or collagen gel. The coverslips are placed in a plastic or glass test tube with culture medium, and the tubes are rotated at  $\sim$ 12 rev/h in an ambient air incubator warmed to 36°C. Filter inserts are placed in multiwell plates containing culture medium, the tissue slices are placed on the membrane, and the cultures are grown in a CO<sub>2</sub> incubator. See text for details.

Once the thrombin is added, the tip of the pipet is used to gently mix the solutions and to position the tissue near the center of the coverglass. The Petri dish is covered to prevent evaporation. Within 10–15 min, the plasma should form a clot, thus securing the tissue to the coverglass.

Collagen gels provide an alternative approach for attaching tissue slices to coverglass. Aqueous solutions of collagen are commercially available (Vitrogen 100, Collagen Corp., Palo Alto, CA), and the manufacturer provides detailed methods of collagen gel preparation for use in cell and tissue culture work. To promote attachment of the tissues, coverglasses are cleaned and prepared as described above. Even with such coverglass cleaning protocols, it may be desirable or necessary to apply a layer of poly(L-ornithine) (P-ORN) or air-dried collagen to the coverglass to promote attachment of the encapsulating gel to the glass. P-ORN solution (1 mg/ml in 10 mM sodium borate buffer) is applied to cover the top surface of coverslips for 1 h, then rinsed off with sterile water and allowed to air-dry in a sterile environment. A small amount (10  $\mu$ l) of collagen solution (2.4 mg/ml) is applied to the coverslips and allowed to air-dry. When dry, any residual salt is washed off with filter-sterilized phosphate-buffered saline (PBS). The coverslips are kept hydrated until ready for use, at which time the PBS is removed, tissue is placed on the coverslips, and collagen solution (25  $\mu$ l) is added to cover the tissue slices. Large Petri dishes with coverslips are then placed in a warm (36°C), humidified incubator for 1–4 h to promote gelation of the collagen.

When the slices are firmly attached to the coverslips, they are placed in individual glass culture tubes (16  $\times$  150 mm) with 1 ml of culture medium. The medium is composed of 50% minimum essential medium (MEM) without phenol red (Gibco), 25% horse serum (Gibco), 25% HBSS (Gibco), 6 mg/ml dextrose (Sigma), and 25 mM Hepes (Sigma), with the pH adjusted to 7.3. The tubes are placed in a modified roller drum (Glas-Col, Model RD4512) tilted at an angle of about 5° with respect to horizontal, and which rotates at approximately 12 rev/h to facilitate aeration of the tissue (7). The cultures are maintained in ambient air in a warmed (36°C) environment.

## 2. Filter Cultures

Filter cultures provide a means for short- or long-term culturing of brain tissue without the need to physically rotate the tissues or attach them to coverglass. Tissues are prepared as described above, then placed on cell culture inserts with polyethylene terephthalate (PET), track-etched porous membranes (0.4 or 1.0  $\mu$ m in pore size). In conjunction with six-well tissue culture plates (Falcon No. 3502), we use culture inserts that are translucent (Falcon No. 3090 or 3102) to facilitate periodic inspection of the cultured tissues using a

dissecting microscope. Culture medium (1 ml) containing 50% MEM, 25% HBSS, 25% horse serum, 2 mM glutamine, and 0.044% NaHCO<sub>3</sub> (10) is added to each well. One or more tissue slices are then positioned on each membrane. The filter cultures are maintained in an incubator (Heraeus) containing warmed (36°C), humidified air with 5% CO<sub>2</sub>.

## 3. Comparison of Roller Tube and Filter Cultures

Each type of tissue culture technique has its advantages. 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 (8, 11), 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 live tissue imaging, since the slices are securely attached directly to a piece of glass that can serve as the microscope coverslip. For live tissue imaging, a more complicated scheme is required for mounting tissues in a way that permits on-stage perfusion of solutions (12).

## C. Fluorescent Staining

### 1. Labeling Microglia with FITC-IB<sub>4</sub>

For fluorescent staining of microglia in living or fixed brain tissues, a fluorescein isothiocyanate (FITC)-conjugated isolectin B<sub>4</sub> is prepared from the seeds of *Griffonia simplicifolia*. This fluorescently labeled lectin (FITC-IB<sub>4</sub>) is available commercially as a lyophilized powder (Sigma; Product No. L-2895). GSA-IB<sub>4</sub> (also known as BSI-B<sub>4</sub>, or simply IB<sub>4</sub>) has an exclusive affinity for terminal  $\alpha$ -D-galactosyl residues (13), and in brain tissue IB<sub>4</sub> appears to selectively label microglia and endothelial cells lining blood vessels (14).

To label microglia in brain tissue slices, a stock solution of FITC-IB<sub>4</sub> is prepared in water at 200  $\mu$ g/ml and stored frozen (–20°C) as aliquots (200  $\mu$ l/aliquot). At the time of staining, an aliquot is thawed, the appropriate amount of stain is removed, and the unused portion of the aliquot is returned to the refrigerator (4°C) for future use. This thawed stock solution, if maintained at 4°C, appears to retain adequate staining potential for at least a week.

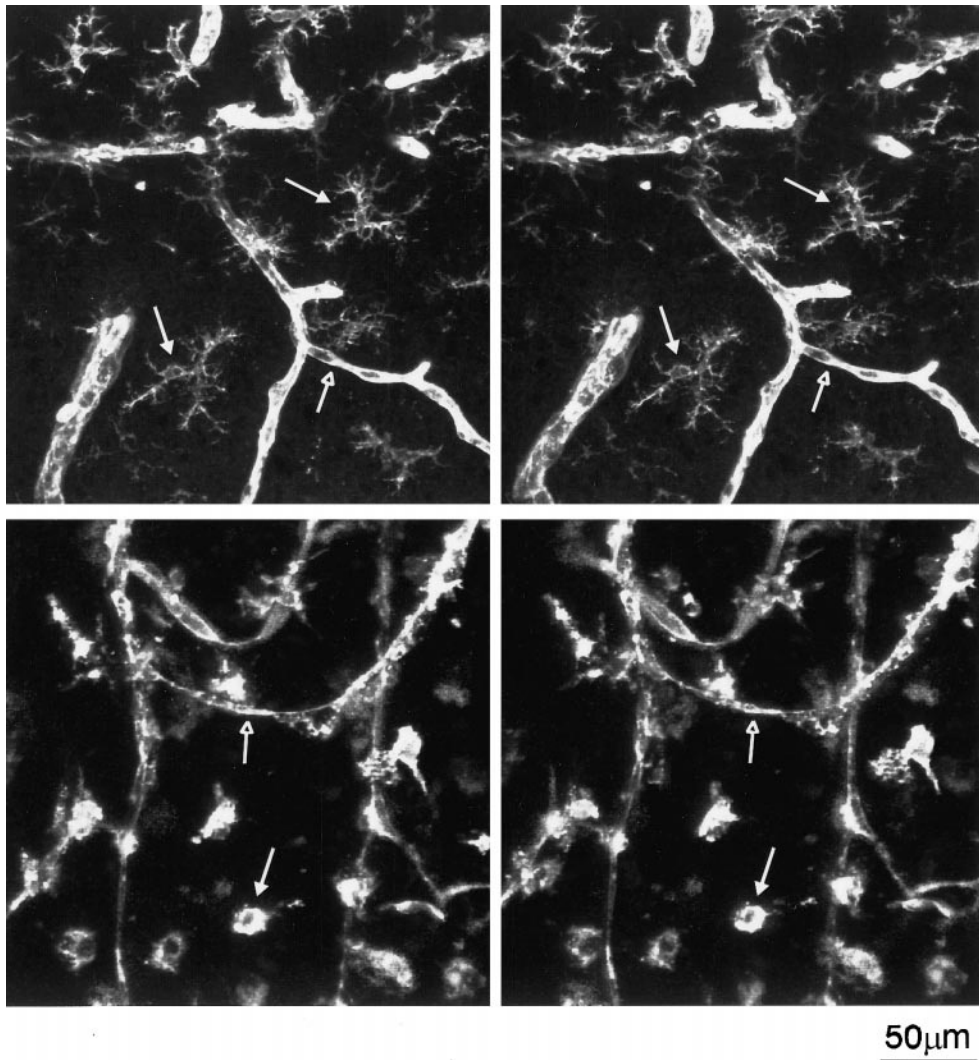
*a. FITC-IB<sub>4</sub> staining of formaldehyde-fixed tissue slices.* For microglial staining in fixed brain tissue, tissue slices are first fixed in 4% paraformaldehyde in PBS or HBSS. After rinsing, tissues are incubated in a working solution of IB<sub>4</sub> that is prepared by diluting the

stock (200  $\mu\text{g/ml}$ ) 40-fold for a final working concentration of 5  $\mu\text{g/ml}$ . The duration of tissue staining can be adjusted to suit the needs of the study. For thick brain tissue slices, it is often necessary to incubate the tissue for a minimum of several hours to provide adequate staining of cells located deep ( $>50\ \mu\text{m}$ ) within the tissue. Longer incubation times can improve the staining intensity of microglial cells situated at deeper levels. We have not encountered significant background levels of tissue staining even with the longest incubation times (3 days).

*b. FITC-IB<sub>4</sub> staining of fixed and permeabilized tissue slices.* IB<sub>4</sub> staining can be used in conjunction with fluorescent antibody labeling protocols, even

when it is necessary to permeabilize cell membranes with detergent. For example, to investigate the relationship of IB<sub>4</sub>-labeled cells to other tissue components such as astrocytes, single tissue slices can be labeled with IB<sub>4</sub> and with antibodies against glial fibrillary acidic protein (GFAP). Fixed tissue slices are detergent extracted (1% Triton X-100 in PBS for 24–48 h), blocked with NH<sub>4</sub>Cl (50 mM) and 20% horse serum, then incubated in primary and secondary antibodies. Since IB<sub>4</sub> staining persists during detergent extraction, the lectin can be applied either before or after cell membranes are permeabilized.

*c. FITC-IB<sub>4</sub> staining of live tissue slices.* An interesting advantage of the IB<sub>4</sub> staining procedure is the



**FIG. 2.** Stereo pair images of hippocampal tissue slices stained with FITC-IB<sub>4</sub>, which labels both microglia and blood vessels. (Top) A tissue slice was prepared from a P7 rat, immediately fixed, stained, and examined with a confocal microscope. Note staining of highly ramified microglial cells (solid arrows), as well as branched blood vessels (open arrow). The images represent stacks of 16 optical sections collected at 3- $\mu\text{m}$  intervals in the Z dimension. Thus, the apparent depth of view is 48  $\mu\text{m}$ . (Bottom) A tissue slice was prepared from a P7 rat and cultured for 1 day. Note the change in morphology of microglia (solid arrow) from a highly ramified to more rounded or amoeboid shape. Also, much of the fluorescent dye appears to be internalized such that the nuclei are apparent as dark spheres. At this point in culture, the blood vessels have begun to collapse.

possibility of using the fluorescent lectin as a surface marker of live microglial cells. We have found that FITC-IB<sub>4</sub> can be used to label microglia in live rat brain tissue slices, and that under appropriate conditions the dynamic movements of the fluorescently labeled microglia can be imaged by time-lapse confocal microscopy with no apparent detrimental effect on cell structure or motility. Live cultured tissue slices are stained with FITC-IB<sub>4</sub> by adding stock solution to the culture medium at a final concentration of 5 μg/ml. In the case of roller tube cultures, 25 μl of stock solution (200 μg/ml) is added directly to the 1 ml of culture medium in the roller tube, and the cultures are returned to the incubator (36°C). To allow for the stain to penetrate the tissue, the dye is applied for a minimum of 45 min before the tissue is rinsed by two to three exchanges with dye-free culture medium over 5 to 10 min. Cultures are then mounted for microscopic examination. Staining of filter membrane cultures is equally effective, especially when some of the dye-containing solution is added directly onto the tissue slices.

When FITC-IB<sub>4</sub> is used to stain live tissue slices, microglia cells become labeled in a pattern that shows intracellular labeling of organelles as well as surface membrane labeling. This pattern of labeling is likely due to the high rate of turnover of the microglial surface membrane, which results in internalization of the fluorescent label. Incubation of live tissue slices in FITC-IB<sub>4</sub> appears not to be detrimental to the microglia. Even when the fluorescent lectin is maintained in the cultures for several days, microglial cells continue proliferating and moving about. Examples of IB<sub>4</sub> staining and three-dimensional structure of microglia at the time of brain slice preparation and one day later are shown in Fig. 2. The dynamics of IB<sub>4</sub>-stained microglial cell division are shown in Fig. 3.

## 2. Fluorescent Nucleic Acid Dyes for Visualizing Live and Dead Cell Nuclei

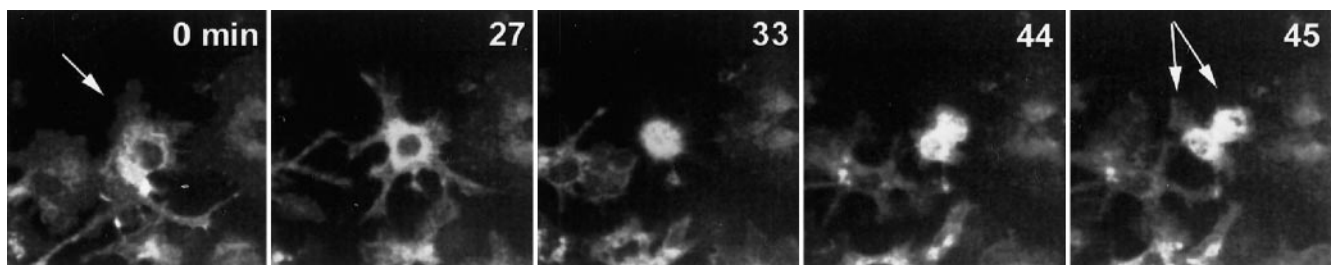
New visible-wavelength fluorescent nucleic acid stains for labeling live and dead cells have recently become available (Molecular Probes). These stains

have varying affinities for binding cellular DNA and RNA, and those stains that have a comparatively higher affinity for binding DNA over RNA are potentially useful for labeling the nuclei of cells in brain tissue slices. Nuclear staining is preferable to cytoplasmic staining of cells in thick tissues because individual cell nuclei can be distinguished from one another. Some of the dyes are cell permeant and therefore should label live cells as well as dead cells, whereas other dyes are membrane impermeant and should selectively label cells with a compromised plasma membrane. We have screened several of the commercially available dyes to determine which of them may be useful for staining cells in brain tissue slices. Other workers have successfully stained cells in brain tissue slices using these or other dyes (15–17). We have been especially interested in the visible-wavelength dyes because of their potential use with laser-based confocal microscopy. We found that the green fluorescent dyes SYTO 11 and SYTO 16 and the red fluorescent dyes SYTO 59 and 61 have nuclear staining properties that make them good candidates for use on brain tissue slices. Moreover, their spectral properties (Table 1) are well matched to the argon and He–Ne (or Ar/Kr) lasers, respectively, which are available on many confocal microscope systems.

To stain brain tissue slices, SYTO dyes are being used at concentrations up to 15 μM in culture medium or HBSS. One of the problems we have encountered when staining tissue slices with nuclear dyes is poor penetration of the dye into the tissue. This situation often leads to intense staining of cells near the surface of the tissue, but inadequate staining of deeper cells. This issue needs to be considered when trying to assess the three-dimensional distribution of live and/or dead cells in a tissue slice. Longer incubation times can improve dye penetration in some instances.

## 3. Visualizing Live and Dead Cells Simultaneously

Sytox Green (Molecular Probes, No. S-7020) is a cell membrane-impermeant nucleic acid stain that readily penetrates thick tissue slices. We have obtained suit-



**FIG. 3.** Time-lapse sequence of live, FITC-IB<sub>4</sub>-stained microglia derived from a hippocampal slice 2 days in culture. A single microglial cell (arrow at 0 min) progresses through cell division to generate two daughter cells (arrows at 45 min). Due to internalization of FITC-IB<sub>4</sub>, the nuclei of the live cells stand out in negative relief. The labeled cells do not seem to be adversely affected by the live fluorescent labeling and imaging procedures. Original images were collected at 2-min intervals.

able staining throughout the thickness of tissue slices with Sytox Green at a working concentration of 1  $\mu$ M. Sytox Green can be combined with SYTO staining of live cells to assess the fraction of cells that appear to be dead by virtue of having leaky plasma membranes (Figs. 4A,B).

#### 4. Double-Labeling Tissues with FITC-IB<sub>4</sub> and Nuclear Dyes

Because SYTO dyes come in a variety of colors (i.e., have differing spectral properties), it is possible to combine a cell type-specific green dye such as FITC-IB<sub>4</sub> and a red general cell marker such as SYTO 61. We have found that staining brain slices with the SYTO nucleic acid dyes reduces subsequent labeling of microglia with FITC-IB<sub>4</sub>. Therefore, for purposes of double labeling, we stain tissues with FITC-IB<sub>4</sub> prior to application of the SYTO dye. This staining sequence seems to preserve the full staining potential of the dyes (Fig. 4C, E).

#### 5. Marking Endocytic and Phagocytic Cells with Fluorescent Lipids

DiIC<sub>18</sub> and similar fluorescent carbocyanine lipids are widely used to label the surfaces of both living and fixed neurons (18, 19). In live cells, turnover of the surface membrane results in internalization of the fluorescent dye. Since activated microglia are highly endocytic and phagocytic, they are readily labeled by application of one of a variety of fluorescent markers from the DiI family (Fig. 4D). In some cases, microglia can be labeled indirectly when they phagocytose other cells that were previously labeled with a fluorescent dye such as DiIC<sub>18</sub>, 4-Di-10-Asp (20, 21), or Mini Ruby (22, 23).

There exist a variety of approaches for labeling highly endocytic and phagocytic cells in live tissue slices. These include (a) soaking the tissue in dye-containing medium; (b) injecting the dye solution directly into the tissue using a pipet; and (c) stabbing the tissue with a dye-coated glass needle or pipet. Soaking tissue with a fluorescent membrane dye such as DiI can produce extensive labeling of the tissue surface, where reactive astrocytes and activated microglia are concentrated (11, 24). Typically, a few microliters of DiI in solvent (20 mg/ml in dimethylformamide or ethanol) is added to 1 ml culture medium or isotonic sorbitol. Addition of 5  $\mu$ l of a stock solution of pluronic [25% in warmed dimethyl sulfoxide (DMSO)] may help reduce formation of dye precipitate, but in our experience this is not completely avoidable.

Fluorescent lipid dyes may also be injected into tissues in an effort to reduce surface staining and improve labeling of cells deep within the tissue, away from the cut tissue surfaces. A stock solution of DiI (0.5%) in *N,N*-dimethylformamide (DMF) is injected through a glass micropipet pulled to a tip diameter of 3–5  $\mu$ m. The dye solution is pressure injected into the tissue using a pneumatic picopump (World Precision Instruments). DMF is among the best solvents for DiI, but even this produces precipitate that clogs the pipet and presents a significant challenge.

As an alternative approach, tissue may be stabbed with a glass micropipet or similar implement coated with the fluorescent probe. This has the advantage of a highly localized delivery of the fluorescent probe with little contaminating spread of dye in solution, which can increase background and reduce image contrast. Glass micropipets pulled to a tip diameter of 1–10  $\mu$ m are coated with DiI by dipping several times in a saturated solution of DiI in ethanol. Dye crystals precipitate onto the outer surface of the pipet, depositing a

**TABLE 1**  
Fluorescent Probes Used to Stain Cells in Live Brain Tissue Slices

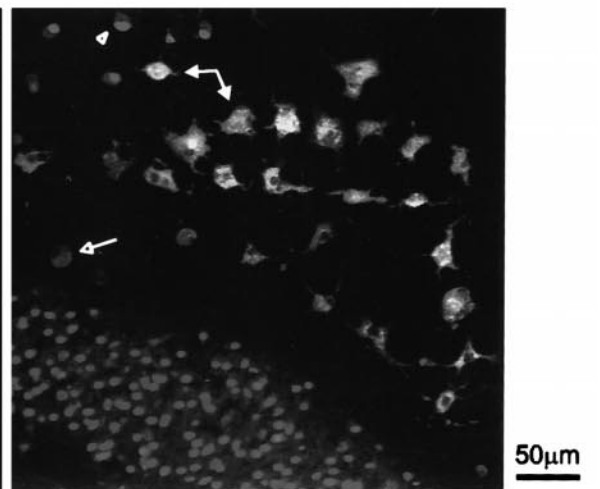
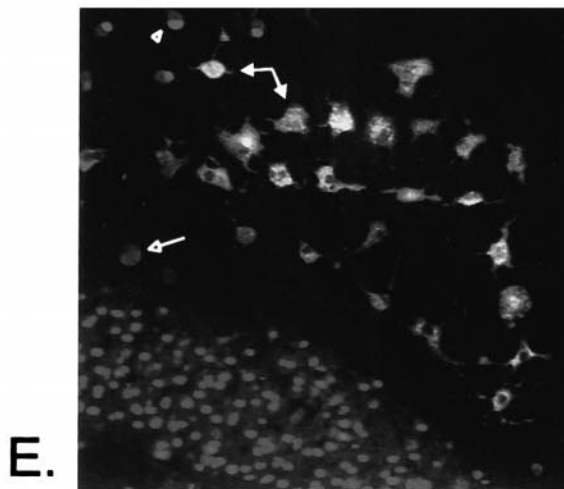
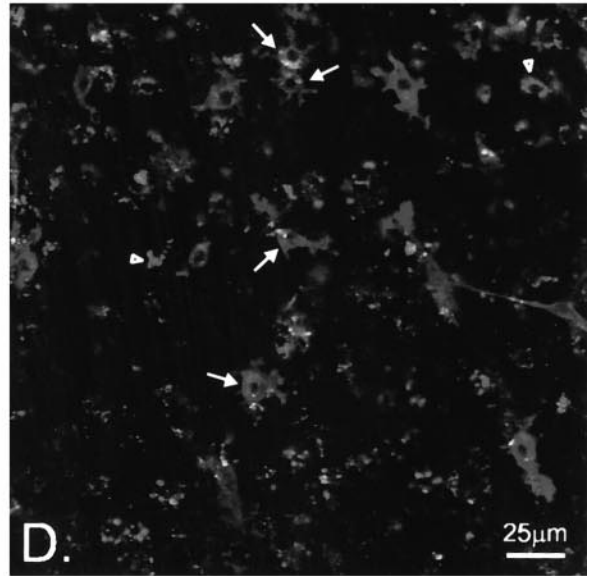
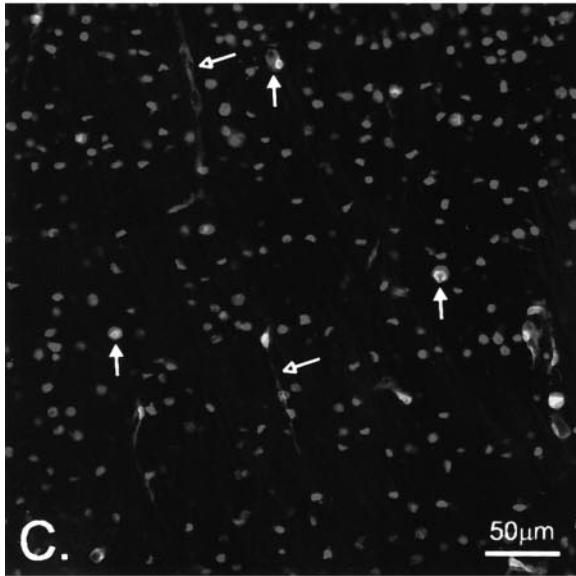
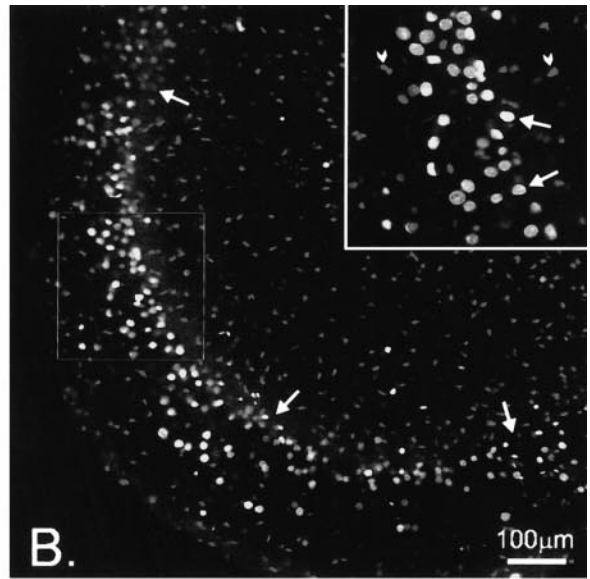
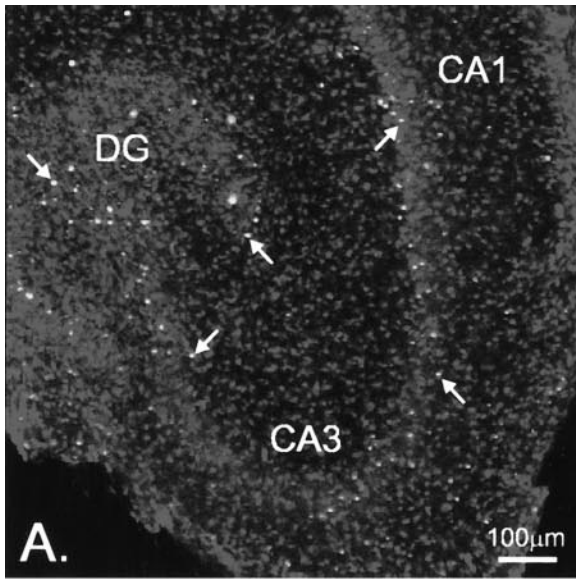
Fluorescent dye	Source	Tissue staining properties	Spectra <sup>a</sup>	Laser <sup>b</sup>
FITC-IB <sub>4</sub>	Sigma Chemical Co.	Microglia; blood vessels	490/520	Ar or Ar/Kr
Sytox Green	Molecular Probes	Nuclei of dead cells	504/523	Ar or Ar/Kr
SYTO 11	Molecular Probes	Nuclei of live and dead cells	508/527	Ar or Ar/Kr
SYTO 16	Molecular Probes	Nuclei of live and dead cells	488/518	Ar or Ar/Kr
SYTO 59	Molecular Probes	Nuclei of live and dead cells	630/645	HeNe or Ar/Kr
SYTO 61	Molecular Probes	Nuclei of live and dead cells	628/645	HeNe or Ar/Kr
DiI <sup>c</sup>	Molecular Probes	Cell surface; endocytic organelles	549/565	Ar or Ar/Kr
DiD <sup>d</sup>	Molecular Probes	Cell surface; endocytic organelles	644/665	HeNe or Ar/Kr

<sup>a</sup> Spectra indicate absorption and emission wavelength peaks in nanometers. For FITC-IB<sub>4</sub>, the spectra reported are for free fluorescein and have not been measured directly for the FITC-conjugated lectin. For SYTO dyes, the absorption peaks correspond to values when dye is bound to DNA (source: Molecular Probes product information sheets).

<sup>b</sup> Ar, argon laser (488 nm); Ar/Kr, mixed-gas argon/krypton laser (488, 568, and 647 nm); HeNe, helium–neon laser (633 nm).

<sup>c</sup> DiIC<sub>18</sub> (3).

<sup>d</sup> DiIC<sub>18</sub> (5).



coating of small dye crystals that can come into contact with cells when inserted into the tissue. A micromanipulator is useful for controlled stabbing of the tissue. The tip of the pipet may be broken off and left behind, as well. In these circumstances, motile phagocytic cells in the area of the insult begin to internalize the dye and are thereby effectively labeled.

#### D. Microscopy Considerations

Fluorescence confocal microscopy is being used to capture the three-dimensional structure and dynamics of fluorescently stained cells in tissue slices. The details of a confocal imaging setup suitable for time-lapse imaging have been described elsewhere (1, 12). Currently, we use a scanning laser confocal microscope equipped with two fluorescence detection channels and a transmitted light channel (Leica TCS NT; Leica, Inc.). Illumination is provided by at least one of three separate lasers: argon (Ar, 15 mW), krypton (Kr, 15 mW), or helium–neon (HeNe, 5 mW). For conventional (single-photon) confocal imaging of live specimens, it is essential to maintain the illumination duration and intensity at a very minimum to reduce or prevent photodynamic damage (12). Often, the health of fluorescently labeled cells will begin to deteriorate well before photobleaching becomes obvious. Probably the single greatest way to promote the success of confocal time-lapse imaging experiments is to operate with the confocal pinhole aperture in an open configuration. Although some spatial resolution will be sacrificed, especially in the axial dimension, the signal-to-noise ratio of the image will greatly improve for a given level of incident laser illumination. One can then lower the incident illumination intensity and thereby reduce the potential for phototoxic effects on the cells.

*Dual-channel imaging.* Many commercially available confocal microscopes have the capacity to image

more than one fluorescent channel at a time, permitting simultaneous acquisition of spatial information for different cell types or different structural or functional aspects of cells. However, when attempting to determine the spatial relationship of distinct structures labeled with different fluorophores, it must be kept in mind that many microscope objectives do not completely correct for axial chromatic aberrations. Thus, the focal distance of the objective could be different for different wavelengths of light, and this will translate into misregistration in the axial ( $Z$ ) dimension such that a single two-dimensional image of double-labeled tissue may contain information from two different focal planes. The extent of the focus difference can be up to 10  $\mu\text{m}$  or more for some low magnification objectives when imaging widely across the visible spectrum (e.g., FITC and Cy5 double label).

The possible effects of chromatic aberration must be considered when working with multilabeled specimens at high spatial resolution. For example, Fig. 4B shows a typical pattern of staining for a brain slice culture that was double-labeled with Sytox Green and SYTO 61. The color image was generated by digitally combining two separate images, one collected in the green channel (Sytox Green) and one in the red channel (SYTO 61) of a two-channel confocal imaging system. Live cells in the tissue are expected to be labeled with the membrane-permeant dye SYTO 61 (red), but to exclude the membrane-impermeant dye Sytox Green (green). On the other hand, dead cells (with leaky membranes) are expected to be labeled with both Sytox Green and SYTO 61, thus appearing yellow. Although one might expect to find only red (alive) or yellow (dead) cells, in fact it can be seen that the nuclei of cells (especially large cells in the pyramidal cell layer) show a range of colors: red, orange, yellow, and

**FIG. 4.** Double or triple labeling of cultured brain slices. (A) Hippocampal slice 2 days in culture stained with the nucleic acid dyes Sytox Green and SYTO 61. Sytox Green is membrane impermeant and thereby stains only nuclei of dead cells. SYTO 61 is membrane permeant and stains both live and dead cells. Thus, live cells should appear red and dead cells should appear yellow. In this particular slice, the vast majority of cells in all areas of the slice, both within and outside of the primary neuronal cell body layers, appear to be viable. Only a few cells are yellow (e.g., arrows). This image is a composite of 12 optical sections spanning 120  $\mu\text{m}$  in the  $Z$  dimension. (B) Sytox Green and SYTO 61 double labeling of another tissue slice showing variations in the pattern of staining. Cells in the pyramidal cell body layer (arrows) appear red, orange, yellow, or green. Such a range of colors could be due in part to chromatic aberration (see text for details). Most of the stained nuclei of cells outside of the pyramidal cell body layer are red, suggesting that they are viable. The inset is a higher magnification view of a portion of the same field, taken at a slightly different plane of focus. Note that many of the nuclei of larger cells (pyramidal neurons) appear green or yellow. Both images represent single optical planes. (C) Three-day-old live hippocampal slice culture double-stained with FITC-IB<sub>4</sub> (green) and SYTO 61 (red). Note the staining of nuclei of FITC-IB<sub>4</sub>-labeled microglia (solid arrows) and other cell types. Such images can yield quantitative information on the density of identified cell types relative to all cells in a given tissue volume. Blood vessels are also stained (open arrow). (D) Two-day-old live hippocampal slice culture double-labeled with FITC-IB<sub>4</sub> (green) and DiD (red). Cells labeled with DiD presumably have a high level of endocytic activity. Note that some of the microglia (arrows) are endocytic at this stage, but other DiD-labeled cells are not labeled with IB<sub>4</sub> (arrowheads). (E) Stereo pair images of a 2-day-old live hippocampal tissue slice triple-labeled with Sytox Green (green), SYTO 61 (red), and FITC-IB<sub>4</sub> (green). The field of view is at the edge of the slice, where some cells have crawled out of the tissue and onto the glass coverslip substratum. These cells include FITC-IB<sub>4</sub>-labeled microglia (solid arrows) and a few nonmicroglial cells (arrowheads). Microglia within the brain slice are also evident (open arrow). Nuclei of dead cells in the pyramidal cell body layer are labeled with Sytox Green. The concentration of SYTO 61 was too low to label live cell nuclei within the tissue, but cells that have crawled out of the tissue are labeled. Reproduced here in black and white. See special color plate section for reproduction in color.

green. This appears to be due at least in part to differences in the focal planes from which the red and the green channels sampled in space. When one focuses slowly through the specimen, it can be seen that many cells appear to change color from red, to orange, to yellow, to green (or vice versa). In one focal plane the cell may appear mostly green, whereas in a slightly different focal plane the cell may appear yellow or orange. This color shift (being readily reversible if one moves the focus back through the sample in the opposite direction) demonstrates the chromatic aberration. When using objectives that are not fully corrected for chromatic aberration, the "red image" of the cell appears in one focal plane and the "green image" of the cell appears in a slightly different plane. Thus, it is important to determine the characteristics of the optical system one is using when attempting to determine precise patterns of colocalization. This can be done using well-defined test specimens such as double- or triple-labeled microspheres (Molecular Probes). Once the optical performance of the system is known, the experimenter can if necessary compensate for the focus difference (e.g., by offsetting the focus by a defined amount for one of the channels) and thereby sample the same volume of tissue in both channels. This will yield images that more accurately reflect the true spatial structure (and state) of cells in the tissue being examined.

## CONCLUDING REMARKS

Techniques for real-time imaging of fluorescently stained tissue slices afford the exciting possibility of studying the dynamics of live cell structure and physiology in the context of a three-dimensional brain tissue architecture. To the extent that cells and subcellular structures of interest can be labeled with specific fluorescent probes, confocal imaging can provide important information on cellular dynamics and cell-cell interactions at high spatial resolution. In some instances, it may be possible to combine information on the dynamics of cells observed in the light microscope with an ultrastructural analysis of the same structures using procedures that convert fluorescent probes to electron-dense products (21). Future work will likely expand on the possibility of combining fluorescent labels of cell structure with indicators of cellular physiology in tissue slices (17). Such multiparameter imaging should improve our understanding of the spatiotemporal aspects of cellular interactions and events that underlie the normal development, function, and pathology of the mammalian central nervous system.

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