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Concepts in Imaging and Microscopy

Exploring Biological Structure and Function with Confocal Microscopy

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Abstract. Confocal microscopy is providing new and exciting opportunities for imaging cell structure and physiology in thick biological specimens, in three dimensions, and in time. The utility of confocal microscopy relies on its fundamental capacity to reject out-of-focus light, thus providing sharp, high-contrast images of cells and subcellular structures within thick samples. Computer controlled focusing and image-capturing features allow for the collection of through-focus series of optical sections that may be used to reconstruct a volume of tissue, yielding information on the 3-D structure and relationships of cells. Tissues and cells may also be imaged in two or three spatial dimensions over time. The resultant digital data, which encode the image, are highly amenable to processing, manipulation and quantitative analyses. In conjunction with a growing variety of vital fluorescent probes, confocal microscopy is yielding new information about the spatiotemporal dynamics of cell morphology and physiology in living tissues and organisms. Here we use mammalian brain tissue to illustrate some of the ways in which multidimensional confocal fluorescence imaging can enhance studies of biological structure and function.

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Introduction

Marvin Minsky's quest to understand how the brain works led him to develop the first confocal microscope (Minsky, 1961, 1988). He reasoned that to make sense of the seemingly insuperable complexity of neural tissue, one needed a microscope that could resolve the fine details of neural structure. Indeed, the structural complexity of brain tissue has provided a formidable challenge to optical microscopy, and the problems are compounded if the physiological aspects of cells and tissues are also considered. A major problem is that conventional (widefield) microscopy of brain tissue—or any other thick biological sample—often yields blurred, low-contrast images in which the fine details of cell structure are obscured. This results primarily from the scatter of light due to interaction with the specimen, and from contaminating light from out-of-focus optical planes. Minsky's solution to these problems was to (a) brightly illuminate only a single spot in the tissue at a time; (b) reject light from out-of-focus regions of the specimen by using a (confocal) pinhole aperture positioned where the objective focused light from the brightly illuminated spot, and (c) scan the sample relative to the illuminating spot to generate a 2-D image of the specimen (see Lichtman, 1994). Thus, he developed a new optical technique—confocal microscopy—which yields substantially improved image contrast and clarity in thick samples. An important feature of this system was the ability to *optically section* through the thick specimen, that is, to systematically collect 2-D images from different levels of depth in the tissue with the same high contrast and clarity.

There are currently a variety of confocal systems, with differing configurations and capabilities, that all operate on

the principle of illuminating a small portion of the tissue and restricting the collection of light from a rather narrow focal plane in the sample. The capacity afforded by these modern confocal microscopes to peer into a relatively thick biological specimen at high spatial resolution has greatly impacted neurobiology and many other areas of biological investigation. Thus, confocal microscopy is enjoying wide application in the biological sciences, enabling studies from the molecular level to that of the whole organism (Summers *et al.*, 1993; Lichtman, 1994; Gard *et al.*, 1995; Turner *et al.*, 1996; Pedley, 1997; Hepler and Gunning, 1998).

Dissecting the 3-D Structure of Tissue

As alluded to above, confocal microscopy provides a means for describing the 3-D architecture of cells in tissues, including the relationships of cells to each other and to other tissue structures. These capabilities are especially useful for examining the complex structure and organization of mammalian brain tissue, for the complexities can be daunting. In many brain regions, for example, various types of neuronal and glial cells are intermingled; moreover, most of the cells have very intricate morphologies with thin, highly branching, tortuous processes that can extend for distances up to several millimeters. Therefore, confocal microscopy has been used in numerous studies to gain high-resolution information about the structure of neural tissue elements.

The advantages of confocal imaging are several-fold. First, many probes of cell and tissue structure are amenable to light microscopy, especially fluorescence microscopy, and commercially available confocal microscopes can be equipped with multiple light sources of differing wavelengths so that a variety of fluorophores can be excited. Some confocal systems use arc lamp illumination, while many use lasers. Commonly used sources of excitation in laser-based confocal microscopes include argon (Ar; 488 nm and 514 nm), krypton (Kr; 568 nm), argon-krypton (Ar-Kr; 488 nm, 514 nm, and 568 nm), and helium-neon (He-Ne; 633 nm) lasers. Shorter wavelength lasers (Ar; 458 nm) are also available at a substantially higher cost and are thus less frequently found on confocal systems. Nevertheless, most systems can excite a set of fluorescent probes spanning nearly the full range of visible wavelengths.

The availability of a variety of excitation wavelengths on a single microscope setup offers the power and convenience of using combinations of fluorescent probes in a single specimen. For double- and triple-labeling experiments, lasers (Kr and He-Ne) and probes (*e.g.*, Cy5) in the far red are very widely separated for the standard green (FITC) and red (TRITC) dyes, precluding concern for bleed-through or crossover between channels (Sargent, 1994; Wouterlood *et al.*, 1998). Thus, the excitation power can be increased to obtain a better signal-to-noise ratio in each of the channels, providing exceptional multiprobe imaging of samples. An example of a brain tissue sample double-labeled to reveal two different glial cell populations is shown in Figure 1.

An important problem that must be considered in multiprobe imaging is the potential artifact introduced by chromatic (wavelength-dependent) aberration in the imaging system (see Pawley, 1995). In essence, when different structures are labeled with different fluorophores, their *x,y,z* positions will appear to shift relative to one another. The magnitude of this lateral (*x-y*) and axial (*z*) chromatic aberration varies with different microscope systems, particularly with different microscope objectives, but under typical, low-magnification imaging conditions, the apparent displacement can be up to several microns. The chromatic aberration of an optical system can be determined quantitatively with the aid of individual latex microspheres containing two different fluorophores with known, identical distributions (available from Molecular Probes, Eugene, OR). If an *x-y* image of a double-labeled microsphere is collected in each channel, and the images (*e.g.*, red and green images) are superimposed, then the lateral displacement of the red and green representations of the microsphere can be calculated, yielding the value of the aberration. The same procedure can be followed to determine the axial chromatic aberration using the *x-z* scanning feature found on many confocal microscopes. Once these values are known for a given set of imaging conditions, adjustments can be made (by image processing) to compensate for the aberration. In the case of axial chromatic aberration, the focus position may simply be adjusted a defined amount for one of the channels. Thus, as long as potential problems with chromatic aberration are considered and handled adequately, multiple-probe confocal imaging can provide important information on the morphology, distribution, and relationships of different cell populations and tissue structures with good spatial resolution.

A second advantage of confocal microscopy is that rather large regions of intact or semi-intact tissue may be imaged at subcellular resolution. For example, it is quite feasible to collect a single stack of confocal optical sections spanning a tissue volume as large as 0.1 mm^3 (containing perhaps as many as 100,000 cells) that can still be observed with a lateral resolution of 1 μm per pixel. Resolution greater than 1 μm may be achieved, usually with the trade-off of a smaller field of view. In fluorescence confocal microscopy, useful images are usually limited to depths into the sample of less than about 100 μm; the limitation is due in large part to light scatter by the tissue. Use of fluorophores with adsorption and emission spectra in the far red (*e.g.*, Cy5) can significantly improve depth penetration over the standard red and green fluorescent dyes.

Third, the optical sectioning capabilities of a confocal microscope, coupled with the ability to collect through-focus stacks of digital images, make it possible for cells in their native tissue setting to be reconstructed and rendered

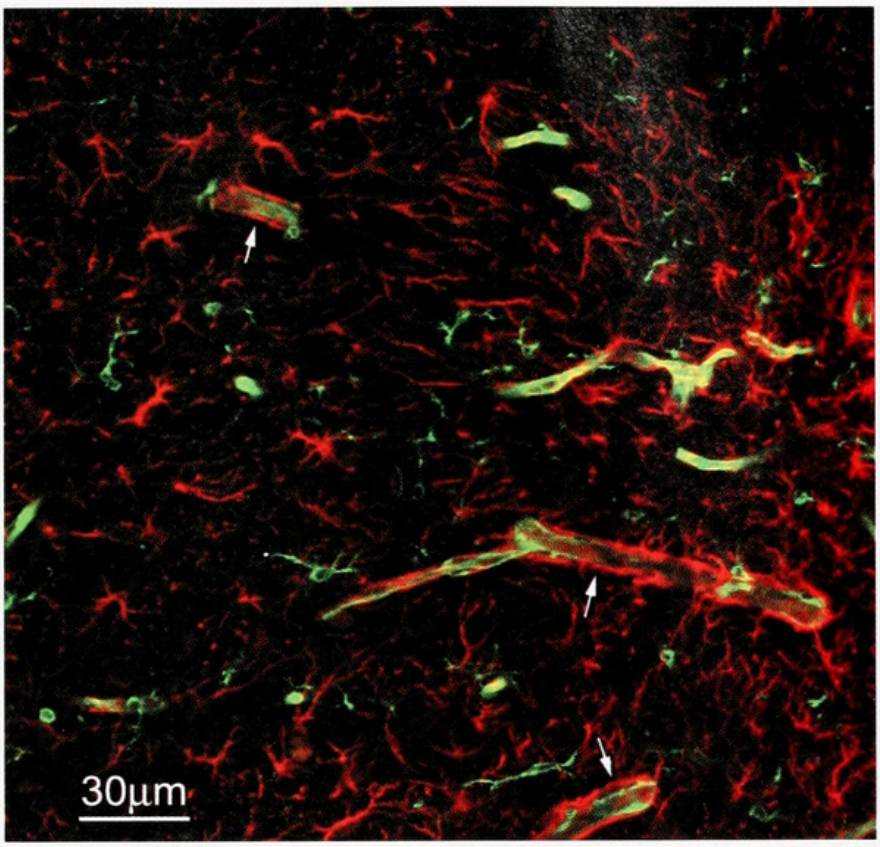

Figure 1. Dual-channel confocal imaging shows the distribution and morphological relationships of microglia (*green*) and astrocytes (*red*) in a double-labeled rat brain tissue slice. Microglia and blood vessels were stained with a fluorescein (FITC)-conjugated isolectin, IB_4 (Sigma, St. Louis; see Dailey and Waite, 1999). Astrocytes were immunohistochemically labeled with monoclonal antibodies against the glial fibrillary acidic protein (GFAP; Sigma, St. Louis, MO), followed by Cy5 conjugated secondary antibodies (Amersham Pharmacia). Note the astrocyte processes (end-feet) that line the surface of the blood vessels (*arrows*).

in three dimensions. One advantage of volume rendering from confocal image stacks is that the sample can be viewed from any angle. This can help clarify the spatial relationships of cells and tissue structures. As an example, we show, in Figure 2, a 3-D rendering of four microglial cells adjacent to a branching blood vessel within a volume of brain tissue reconstructed from a stack of confocal optical sections. It would be very difficult to distinguish the small cellular processes adjacent to the brightly staining blood vessels with conventional widefield fluorescence microscopy.

In most cases, the images generated by the confocal microscope are in digital form. Thus, a wide range of digital image processing capabilities can be applied to the data set. Features of cell and tissue structure can be quantified and analyzed with the aid of software packages that either stand alone or are already integrated into the confocal system software.

Imaging the Dynamics of Cell and Tissue Development and Morphology

In addition to the spatial information about cell structure, we can collect, with time-lapse confocal microscopy, information about the dynamics of cellular structure over time—from seconds to days. In many organisms, cell growth and locomotion play critical roles in tissue morphogenesis during development. Morphological changes may also reflect important responses to changing physiological conditions, as well as to tissue injury.

Among the first applications of time-lapse confocal imaging was a series of studies of the growth of axon terminals within the intact brain of *Xenopus* (O'Rourke and Fraser, 1990; O'Rourke *et al.*, 1994). Similar phenomena have also been amenable to study by time-lapse confocal microscopy in mammalian brain tissue (Smith *et al.*, 1990). For example, the mitotic cycle of neuroblasts (Chenn and McConnell, 1995; Adams, 1996), migration of neurons (O'Rourke *et al.*, 1992; Barber *et al.*, 1993; Fishell *et al.*, 1993; Komuro and Rakic, 1995, 1996, 1998), axonal growth and guidance (Dailey *et al.*, 1994), and the development of dendrites (Dailey and Smith, 1996) have all been imaged by time-lapse confocal microscopy in tissue slices of developing mammalian brain and spinal cord. Time-lapse confocal imaging has also been used to study morphological changes in synaptic structures such as dendritic spines under conditions of physiological plasticity (Hosokawa *et al.*, 1992, 1995). And finally, confocal imaging has been employed to study

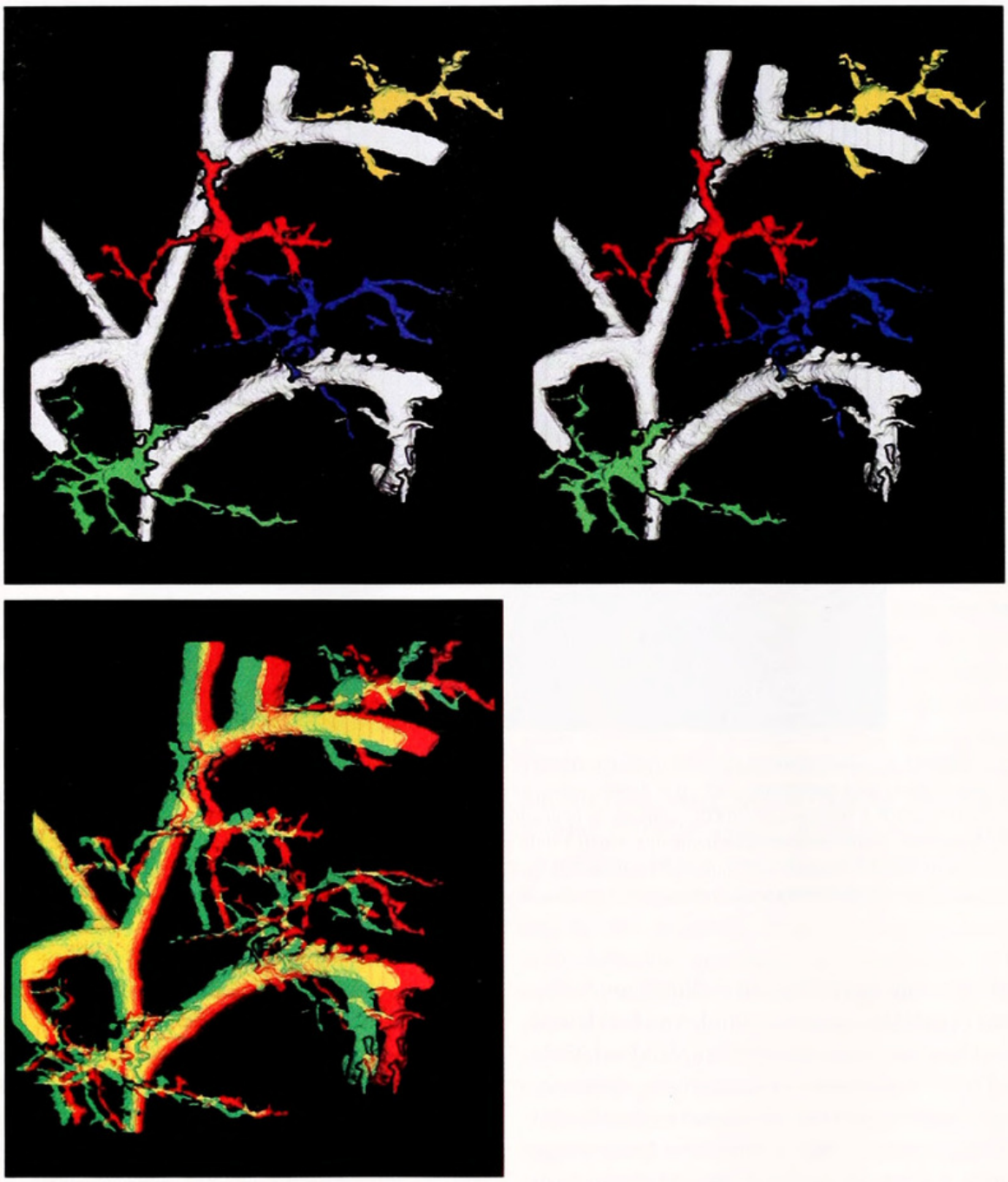

Figure 2. Three-dimensional confocal reconstruction of microglial cells in relation to a branching blood vessel in a rat hippocampal brain slice. A through-focus stack of 93 confocal images was collected at 0.4 μm z-step intervals through a tissue depth of 37 μm. The volume was reconstructed and rendered using Voxblast (Veytek, Fairfield, IA). (Top pair of images) Individual microglial cells were digitally "painted" different colors to distinguish one from another. The left and right images (a stereopair) represent views from slightly different perspectives (offset by 10°) to provide depth information. A 3-D image of the cells can be seen by crossing your eyes as you look at the pair of images. (*Bottom image*) A 3-D image of the same field may be seen using red-blue stereo glasses (red over left eye). Note that some microglial processes appear to contact the surfaces of the blood vessel. An animated movie of this tissue volume is available for viewing on *The Biological Bulletin* Website at <http://www.mbl.edu/html/BB/VIDEO/BB.video.html>.

the dynamics of glial cells responding to neural tissue injury (Brockhaus *et al.*, 1996; Dailey and Waite, 1999). In each case, the dynamic features of cell structure and movement could be viewed in a near-native tissue environment.

One of the under-utilized features of confocal microscopy is the ability to image dynamic cell and tissue structures in four dimensions (4-D); that is, in three spatial dimensions over time (*e.g.*, Kriete and Wagner, 1993; Konijn *et al.*, 1996; Errington *et al.*, 1997; Zimmermann and Siegert, 1998). This can be accomplished by collecting stacks of confocal images at set time intervals The resultant time series of confocal image stacks can be used to reconstruct 3-D views of dynamic cell and tissue development. Mark Cooper's group has elegantly applied this approach to early

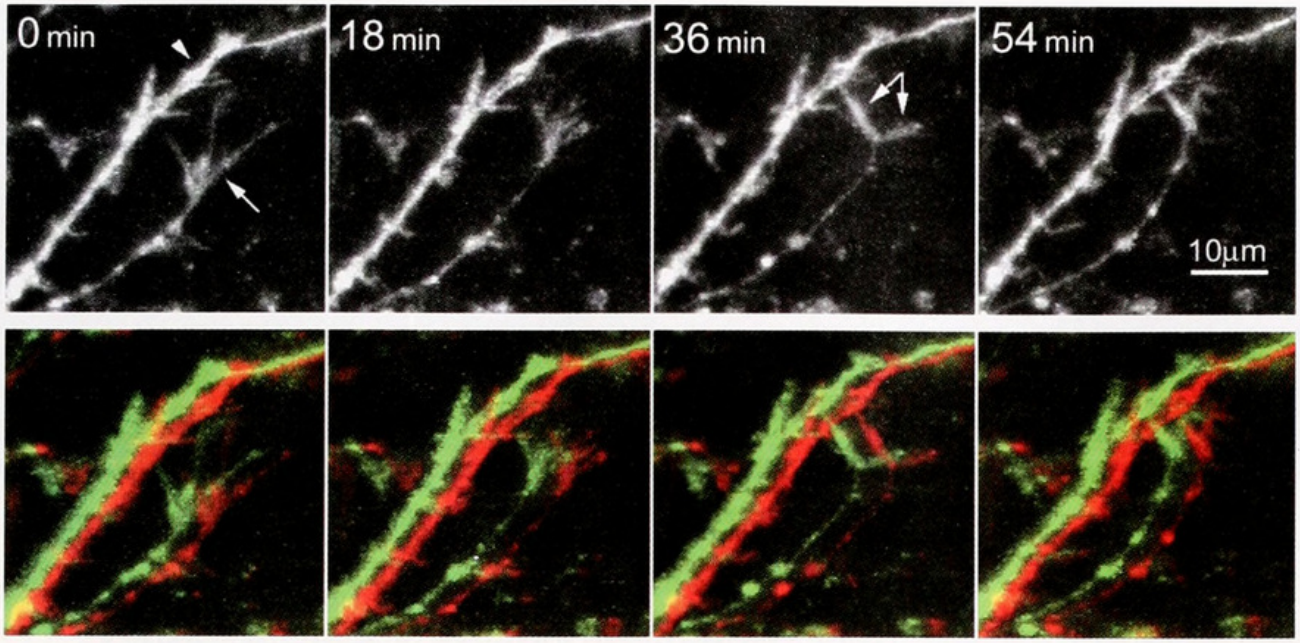

Figure 3. Time-lapse sequence shows the dynamics of axon growth and contact with a dendrite in a developing rat hippocampal slice. Neurons were labeled with a fluorescent membrane dye, DiI. To image growth of neuronal processes in three dimensions, stacks of 16 optical sections spanning 30 μm in the axial dimension (2-μm z-steps) were collected at time intervals of 6 min. Images in the top sequence represent a simple axial projection of the 16 images in the through-focus stack. The bottom series of images are red-green stereo images of the same data to provide depth information (viewing requires red-green or red-blue stereo glasses). A thin axon (*arrow*) extends parallel to a dendrite (*arrowhead*). Note the long thin filopodia at the leading edge of the growth cone (0 min), which advances (18 min) and bifurcates (*arrows,* 36 min). The left branch of the growth cone contacts the adjacent dendrite, and the axon growth is subsequently reoriented in that direction (54 min). A time-lapse movie of the axon growth is available for viewing on *The Biological Bulletin* Website at <http://www.mbl.edu/html/BB/VIDEO/BB.video.html>.

zebrafish development (Cooper, 1999), demonstrating the power of time-resolved, 4-D confocal imaging in a fully intact, experimental vertebrate preparation. In Figure 3 we illustrate the use of 4-D confocal imaging to capture the dynamic behavior of an axonal growth cone extending and contacting a dendrite within a rat hippocampal brain slice.

Imaging Cell and Tissue Physiology

With increasing frequency, it is becoming necessary—and feasible—to gather information about both the structure and physiology of the biological specimen. This is especially essential for studies on neural tissue, where spatial and temporal patterns of electrical and chemical signals play critical roles in brain function. Optical imaging of the physiology of individual cells within the context of a 3-D tissue can provide a powerful means of exploring tissue organization and function. Within a single field of view, the activity of many tens or hundreds of cells may be observed simultaneously. This can help elucidate physiological features of populations of cells, reveal distinct functional properties and relationships of different cell types, and define functional domains within a tissue.

In conjunction with the various fluorescent probes used in cell physiology, confocal imaging can provide information on absolute values of, as well as transient changes in, membrane potential, pH, intracellular calcium, and several other ions and physiological factors. For example, fluorescent calcium indicator dyes (such as fluo-3) have been used often to investigate the dynamics of intracellular calcium fluctuation in a variety of cell and tissue preparations. Such studies have helped define the spatiotemporal aspects of intra- and inter-cellular calcium signals (Cornell-Bell *et al.,* 1990; Cleemann *et al.,* 1998; Wier *et al.,* 1997). Confocal physiological imaging also has been feasible for studies in thick brain tissue slices (Dani *et al.,* 1993; van den Pol *et al.,* 1992; Dailey and Smith, 1994; Komuro and Rakic, 1996; Guerineau *et al.,* 1998) and in other complex neural preparations, such as the intact zebrafish (Cox and Fetcho, 1996) and the neuromuscular junctions of frog (Reist and Smith, 1992) and fly (Karunanithi *et al.,* 1997). Figure 4 illustrates the use of confocal imaging to examine, in cultured brain tissue, the spatiotemporal patterns of intra- and inter-cellular activity in neuroglial cells in response to a physiological perturbation.

Many calcium imaging experiments that use laser confocal microscopy have employed nonratiometric calcium indicator dyes (*e.g.,* fluo-3, calcium green), primarily because the most popular ratiometric dyes (fura-2 and indo-1) re-

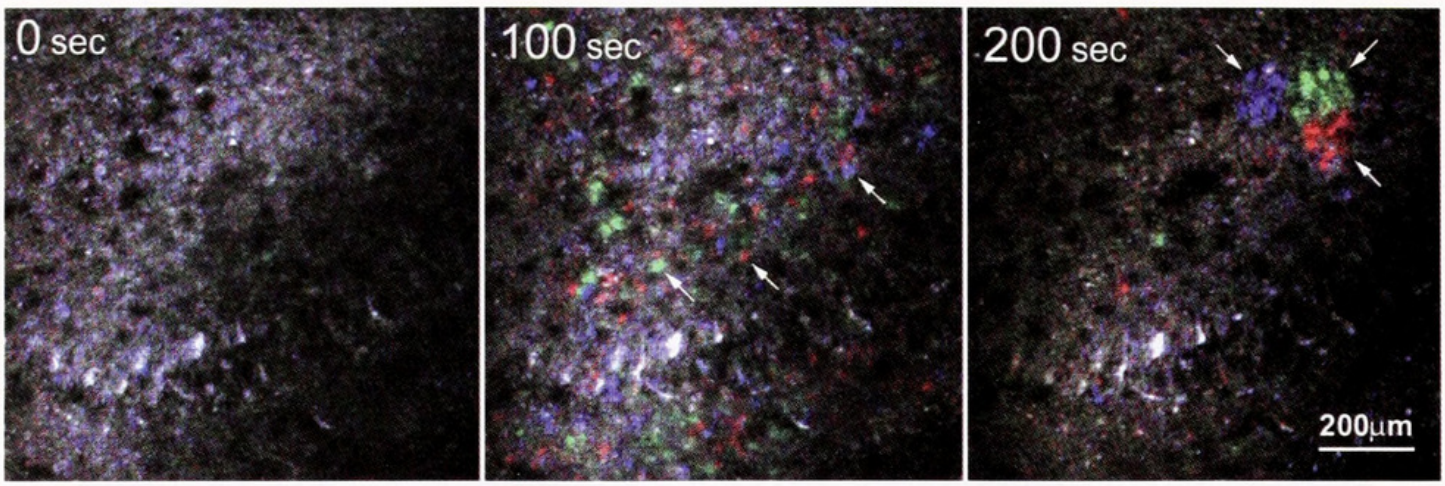

Figure 4. Physiological time-lapse imaging reveals changing spatiotemporal patterns of intracellular calcium (Ca^{2+}) activity in brain tissue in response to potassium (K^+) depolarization. The slice was loaded with fluo-4 AM (Molecular Probes, Eugene, OR), a membrane-permeant fluorescent indicator of intracellular calcium, and mounted in an open chamber for imaging. Single confocal scans were collected at 7-s intervals to detect changes in fluorescence intensity, which reflect changes in intracellular calcium levels. Each panel (left, center, right) is a composite of three images acquired at three slightly different time-points (7 s apart) and encoded red, green, or blue. Thus, the colors represent points in time when cells are active. Inactive cells appear black, and cells with sustained high calcium levels appear white. The left panel, corresponding to a time-point prior to K^+ depolarization, shows a low level of spontaneous calcium activity (few colored cells). The center panel, taken just after addition of medium containing high (9 m*M*) K^+, shows a much higher level of calcium activity in cells. Note that the small colored patches (corresponding to individual, active cells; *arrows*) are dispersed across the field of view. In the right panel, taken about 100 s later, the isolated cell activity has diminished, and a new pattern of activity emerges corresponding to groups of 5–15 synchronously active cells within patches that are 100–200 μm in diameter. The active cells are probably astrocytes, and the emergence of synchronously active groups of neighboring cells probably represents electrical (gap junction) coupling among astrocytes (Charles, 1998; Harris-White *et al.,* 1998). A time-lapse movie is available for viewing on *The Biological Bulletin* Website at <http://www.mbl.edu/html/BB/VIDEO/BB.video.html>.

quire excitation wavelengths in the ultraviolet (UV) range. Such short-wavelength lasers are expensive and thus less widely available; moreover, chromatic aberration problems associated with UV excitation make confocal microscope design very challenging (Blinton and Lechleiter, 1995). However, several studies have shown that ratiometric physiological data can be obtained by visible wavelength confocal imaging. These studies utilize two calcium-sensitive dyes (fluo-3 and fura red) simultaneously (Lipp and Niggli, 1993, 1994; Schild *et al.,* 1994), or a calcium sensitive (fluo-3) and a calcium-insensitive (rhodamine) dye in combination (Stricker, 1996).

Since many physiological events occur on a very fast time-scale, an imaging system must sample at a sufficiently high rate to resolve such events. Many of the early confocal systems were severely limited by the rate at which they were able to collect and store images. This limitation is increasingly being overcome in two ways. First, with standard laser scanning confocal microscopes, the sampling rate can be increased by reducing the size of the field over which image data is collected. In the extreme case, the "field size" is reduced to a single line that can be repeatedly scanned at high rates (>300 Hz), a so-called line-scanning mode. This yields limited spatial information, but provides the extremely high time-resolution necessary for resolving fast physiological events such as neural synaptic activity (Schild *et al.,* 1994; Korkotian and Segal, 1998; Yuste *et al.,* 1999).

Second, several video rate or "real-time" confocal systems have been developed, some of which are capable of collecting over one hundred *x-y* (2-D) images per second. These systems have been utilized to study preparations as diverse as individual mesenchymal cells (Vesely and Boyde, 1996), perfused whole rat heart (Hama *et al.,* 1998), kidney (Andrews, 1996), and mammalian nerve and blood vessels *in vivo* (Bussau *et al.,* 1998; Papworth *et al.,* 1998).

While confocal imaging is permitting unprecedented observations of intact tissues and organisms, it is also extending our view into the dynamic subcellular and molecular world. This new capability has been closely coupled with the development and use of vital fluorescent probes such as green fluorescent protein (GFP)-tagged proteins (Chalfie *et al.,* 1994). Such probes can be engineered to target particular organelles, delivered to living cells and organisms, and imaged by time-lapse confocal microscopy, so that the growth, dynamics, and reorganization of subcellular compartments can be studied (Cole *et al.,* 1996; Terasaki *et al.,* 1996).

Outlook

The plethora of cell and molecular probes now available will, with increasing frequency, permit studies aimed at elucidating both morphological and physiological features of biological specimens. An especially exciting aspect of current biological investigation is the ability to assess the spatiotemporal dynamics of molecules in living cells, tissues, and intact organisms. Progress in such studies critically depends upon the availability of imaging tools that provide sufficient spatial and temporal resolution and that are, to the extent possible, noninvasive and nondestructive. We can anticipate that advances in confocal microscopy will continue to play a role in extending our capacity to probe the relationship between biological structure and function, from molecule to organism.

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