19

Confocal Microscopy of Living Cells

Michael E. Dailey, Erik Manders, David R. Soll, and Mark Terasaki

INTRODUCTION

If a picture is worth a thousand words, then a movie may be worth a million words. Microcinematography and, later, video microscopy have provided great insight into biological phenomena. One limitation, however, has been the difficulty of imaging in three dimensions. In many cases, observations have been made on cultured cells that are thin to start with or tissue preparations that have been sectioned.

The development of the first beam-scanning confocal microscope was motivated by the goal of making observations in the tissues of living organisms (Petran *et al.*, 1986). The optical sectioning capability of the confocal or multi-photon (MP) microscope allows one to make thin-slice views in intact cells or even intact animals. Confocal microscopes are now fairly common, and because they employ non-ionizing radiation, they are increasingly being used to study living cells and tissue preparations.

What are the specific challenges of applying confocal imaging to studies of living cells? First, the experimenter must do no harm. Arguably, the main obstacle in living cells microscopy is not "getting an image" but doing so without upsetting the cell. To be useful, the study must be carried out on a biological system that retains normal function and can be subjected to controlled conditions while on the stage of the microscope. Often, environmental variables such as temperature, CO_2, or pH must be regulated and/or an efficient directional perfusion system must be used. Unfortunately, the difficulty of keeping cells alive and functioning on the microscope discourages many researchers. This chapter is designed to help them succeed well enough to become convinced of the importance and utility of this approach.

Other difficulties are more specific to confocal fluorescence microscopy. All studies with fluorescence benefit from collecting as much of the emitted fluorescent light as is possible, but this is particularly important for studies of living cells because photodynamic damage and consequent alteration in normal cell behavior is a very real possibility (see Chapters 16 and 38, *this volume*). Therefore, optimizing microscope photon collection efficiency is crucial for successful confocal microscopy of living cells.

Another difference between living and fixed cell studies is the element of time. All living processes have an inherent time course, and the imaging system must produce images at the appropriate rate to show the changes involved. The amount of light necessary to obtain the data must be apportioned over time so that enough images can be obtained to describe the process under investigation without damaging the cells. Although early confocal microscopes had a relatively slow scan speed, newer technology now permits very rapid image collection to explore spatially and temporally dynamic biological processes (see Chapter 10, *this volume*). In single-beam scanning systems, the field of view often is reduced to achieve higher imaging speeds.

The fluorescent probes used in studies of living cells must not impair normal cell function. Immunofluorescence, which has been used so successfully to localize molecules in fixed cells, has not been practical in living cells. However, there are now many commercially available fluorescent probes for structural and physiological studies of cells and tissues (see Chapters 16 and 17, *this volume*). Of even more importance, the "green revolution" based on the green fluorescent protein (GFP) has changed the landscape and is ushering in an exciting period of biological imaging of proteins in living cells, and of various cell types in living, intact tissue preparations (Chalfie *et al.*, 1994; Bastieans and Pepperkok, 2000).

Although confocal microscopy of living cells is difficult, its usefulness was demonstrated over 15 years ago in two pioneering studies. Cornell-Bell and colleagues (1990) used confocal microscopy to make a major discovery: the existence of glutamate-stimulated, transcellular Ca^{2+} waves in astroglia. In the same year, confocal microscopy was used to characterize developmental changes in an intact animal by imaging neuronal axons and their growth cones in the developing brain of a tadpole (O'Rourke and Fraser, 1990). Ever since these pioneering studies, there has been an increasing use of confocal microscopy to study dynamic processes in an array of diverse biological preparations. When the second edition of this volume appeared in 1995, about 80 papers using confocal microscopy on living cells were found. Today (early 2005), there are over 500 published studies using live-cell confocal imaging.

While live-cell applications of confocal imaging have expanded significantly over the past decade, multi-photon imaging (see Chapters 21 and 28, *this volume*) is poised to make a similar impact on live-cell and tissue studies in the next decade. There are trade-offs, however. MP imaging can be useful especially for very deep penetration in tissues (>100 µm) where non-descanned detection increases signal substantially, but on thinner specimens, the actual damage/excitation may be greater for MP than for single-photon confocal imaging (Tauer, 2002; see also Chapter 38, *this volume*). Moreover, the cost differential is such that one could have two to three graduate students working away on disk scanners or simpler beam-scanning confocal units for every one on a MP unit. At any rate, most of the topics covered in this chapter are relevant for both single-photon confocal and MP excitation.

Michael E. Dailey • University of Iowa, Iowa City, Iowa 52242
Erik Manders • Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands
David R. Soll • University of Iowa, Iowa City, Iowa 52242
Mark Terasaki • University of Connecticut Health Center, Farmington, Connecticut 06032

Handbook of Biological Confocal Microscopy, Third Edition, edited by James B. Pawley, Springer Science+Business Media, LLC, New York, 2006.

OVERVIEW OF LIVING-CELL CONFOCAL IMAGING TECHNIQUES

Although live-cell imaging often involves time-lapse microscopy to monitor cell movements, modern approaches are extending these observations well beyond simply making movies of cell structure. Increasingly, time-lapse imaging is being integrated with specialized techniques for monitoring, measuring, and perturbing dynamic activities of cells and subcellular structures. Below we summarize some major techniques available for studying the dynamic organization of molecules and cells in live biological specimen. These techniques are summarized in Table 19.1.

Time-Lapse Fluorescence Imaging

Time-lapse fluorescence imaging involves repeated imaging of a labeled specimen at defined time points, thereby permitting studies on the dynamic distribution of fluorescently labeled components in living systems. Imaging can be performed in one, two, or three spatial dimensions: one-dimensional (1D) imaging involves rapid and repeated imaging of single scan lines; two-dimensional (2D) imaging involves repeated imaging of single focal planes; and three-dimensional (3D) imaging involves repeated imaging of multiple focal planes in a thick specimen. The time intervals for sequential image collection can range from sub-second to days or even months (e.g., Gan *et al.*, 2003).

Many small molecule, vital fluorescent probes that give highly specific cellular or subcellular patterns of labeling are now available (see below and Chapters 16 and 17, *this volume*). In addition, GFP or GFP-related proteins are now routinely fused to other proteins of interest, and the inherent brightness and photostability of many of these fluorescent proteins make them well suited for the repeated imaging needed for time-lapse studies. Together, these fluorescent probes are affording a seemingly limitless array of possibilities for imaging molecular components in live cells.

Multi-Channel Time-Lapse Fluorescence Imaging

The plethora of excellent vital fluorescent labels with varying spectral characteristics (including spectral variants of GFP) allows multi-label experiments to visualize the relative distribution of several different cell or tissue components simultaneously. Advances in imaging technology have facilitated automated collection of more than one fluorescent channel (either sequentially or simultaneously) with improved ability to maximize signal collection and to separate partially overlapping signals.

In addition to studies using multiple fluorescent tags, multi-channel data collection permits ratiometric imaging of single probes whose spectral properties (absorption or emission) change depending on ionic conditions, such as the Ca^{2+} sensitive physiological indicator, indo-1 (see Chapter 42, *this volume*).

Spectral Imaging and Linear Unmixing

Increasingly, experiments are incorporating multiple fluorescent probes within single cells or tissues to define the differential distribution of more than one labeled structure or molecular species. Such multi-color or multi-spectral imaging experiments require adequate separation of the fluorescent emissions, and this is especially problematic when the spectra are substantially overlapping. Spectral imaging utilizes hardware to separate the emitted light into its spectral components. Linear unmixing is a computational process related to deconvolution that uses the spectra of each dye as though it were a point-spread function at a fixed location to "unmix" the component signals (Tsurui *et al.*, 2000; Lansford *et al.*, 2001; Hiraoka *et al.*, 2002). Although together these analytical tools can be used to discriminate distinct fluorophores with highly overlapping spectra (Zimmermann *et al.*, 2003), they do so at the cost of requiring that significantly more photons be detected from each pixel.

Fluorescence Recovery After Photobleaching

Fluorescence recovery after photobleaching (FRAP), also known as fluorescence photobleaching recovery (FPR), is a technique for defining the diffusion properties of a population of fluorescently labeled molecules (Axelrod *et al.*, 1976; Koppel *et al.*, 1976; for review, see Lippincott-Schwarz *et al.*, 2003). Typically, a spot or line of intense illumination is used to bleach a portion of a fluorescent cell, and the recovery of fluorescent signal back into the bleached area from adjacent areas is monitored over time (usually seconds to minutes). Although this technique can yield quantitative information on the diffusion coefficient, mobile fraction, and binding/dissociation of a protein, care has to be taken not to use so much power in the bleach beam that the cellular structure is disrupted (Bloom and Webb, 1984; Flock *et al.*, 1998; see also Figures 49.10 through 49.14, *this volume*). Quantitative assessments of FRAP data, which can be confounded by uncertainties in the experimental and biological parameters in living cells, may benefit from computer simulations (Weiss, 2004).

Fluorescence Loss in Photobleaching

This technique utilizes repeated photobleaching in an attempt to bleach all fluorophores within a given cellular compartment (Lippincott-Schwarz *et al.*, 2001). Thus, fluorescence loss in photobleaching (FLIP) can be used to assess the continuity of membrane bounded compartments (e.g., endoplasmic reticulum [ER] or Golgi apparatus) and to define the diffusional properties of components within, or on the surface of, these compartments.

Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) is a technique for defining interactions between two molecular species tagged with different fluorophores (Stryer, 1978; Sekar and Periasamy, 2003). It takes advantage of the fact that the emission energy of a fluorescent "donor" can be absorbed by (i.e., transferred to) an "acceptor" fluorophore when these fluorophores are in nanometer proximity and have overlapping spectra (see Chapters 16, 27, and 45, *this volume*).

Fluorescence Lifetime Imaging

This technique measures the lifetime of the excited state of a fluorophore (Lakowicz *et al.*, 1992 and Chapter 27, *this volume*). Each fluorescent dye has a characteristic "lifetime" in the excited state (usually 1–20 ns), and detection of this lifetime can be used to distinguish different dyes in samples labeled with multiple dyes. Fluorescence lifetime imaging (FLIM) can be utilized in conjunction with FRET analysis because the lifetime of the donor fluorophore is shortened by FRET. In fact, FLIM can improve the measurement during FRET analysis because the fluorescence lifetime is independent of the fluorophore concentration and excitation energy (Bastiaens and Squire, 1999; Elangovan *et al.*, 2002;

Chen *et al.*, 2003; Chapter 27, *this volume*). However, the lifetime can be modulated by environmental considerations (e.g., pH, ion concentration), and this change can be used to measure changes in the concentration of certain ions (Lin *et al.*, 2003).

Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) measures spontaneous fluorescence intensity fluctuations in a stationary microscopic detection volume (about 1 fL) (Magde *et al.*, 1974). Such intensity fluctuations represent changes in the number or quantum yield of fluorescent molecules in the detection volume. By analyzing these fluctuations statistically, FCS can provide information on equilibrium concentrations, reaction kinetics, and diffusion rates of fluorescently tagged molecules (Elson, 2001). An advantage of this approach is the ability to measure the mobility of molecules down to the single molecule level and to do so using a light dose orders of magnitude lower than used for FRAP.

Fluorescence Speckle Microscopy

The dynamic growth and movement of fluorescently labeled structures can be difficult to analyze when these structures are densely packed and overlapping within living cells. Fluorescent speckle microscopy (FSM) is a technique compatible with widefield or confocal microscopy (Adams *et al.*, 2003) that uses a very low concentration of fluorescently labeled subunits to reduce out-of-focus fluorescence and improve visibility of labeled structures and their dynamics in thick regions of living cells (Waterman-Storer *et al.*, 1998). This is accomplished by labeling only a fraction of the entire structure of interest. In that sense, it is akin to performing FCS over an entire field of view, albeit with more focus on spatial patterns than on quantitative temporal analysis. FSM has been especially useful for defining the movement and polymerization/depolymerization of polymeric cytoskeletal elements, such as actin and microtubules, in motile cells (Salmon *et al.*, 2002).

Photo-Uncaging/Photoactivation

Photo-uncaging is a light-induced process of releasing a "caged" molecule from a caging group to produce an active molecule (Politz, 1999; Dorman and Prestwich, 2000). A variety of caged molecules have been synthesized and used experimentally, but in some examples cages have been used to mask a fluorophore, inducing a non-fluorescent state. Excitation light of ~350 nm is used to break photolabile bonds between the caging group and fluorophore, thereby uncaging the fluorophore and yielding a fluorescent molecule.

A related technique utilizes genetically encoded, photoactivatable fluorescent proteins, of which there are currently about a dozen (for review, see Patterson and Lippincott-Schwartz, 2004). Two examples include a photoactivatable (PA) form of GFP, called PA-GFP (ex/em: 504/517), which shows a 100-fold increase in fluorescence following irradiation at 413 nm (Patterson and Lippincott-Schwartz, 2002), and Kaede (ex/em: 572/582), which shows a 2000-fold increase following irradiation at 405 nm (Ando *et al.*, 2002; see also Figure 8.37, *this volume*).

An extension of the photoactivation approach, termed reversible protein highlighting, has been developed (Ando *et al.*, 2004). This involves reversible, light-induced conversion of a coral protein, Dronpa, between fluorescent and non-fluorescent states. One study used this approach to monitor fast protein dynamics in and out of cell nuclei (Ando *et al.*, 2004). Thus, photo-uncaging and photoactivation are complementary to FRAP and can be used in conjunction with time-lapse imaging to mark and follow a population of molecules in order to study their kinetic properties within living cells.

Optical Tweezers/Laser Trapping

Optical tweezers, or single beam laser traps, use the "radiation pressure" of a stream of photons emitted from an infrared laser to "trap" small objects (often a protein-coated bead) and to move them around (Sheetz, 1998; Kuo, 2001; Chapters 5 and 9, *this volume*). This technique has been especially useful for quantifying forces generated by motor protein movement (Ashkin *et al.*, 1990; Block *et al.*, 1990; Kuo and Sheetz, 1993) or the strength of adhesions mediated by cell adhesion molecules (e.g., Schmidt *et al.*, 1993; Baumgartner *et al.*, 2003). Although "laser tweezers" often are used in widefield imaging systems, they also have been incorporated into confocal (Visscher and Brakenhoff, 1991) and MP (Goksor *et al.*, 2004) imaging systems.

Physiological Fluorescence Imaging

The availability of fluorescent physiological indicators extends live-cell confocal and MP imaging studies beyond structural aspects to study cell and tissue physiology (Niggli and Egger, 2004; Rubart, 2004; Wang *et al.*, 2004). Calcium indicators have been the most commonly used physiological probes because calcium is a central signal transduction molecule and in many cell preparations the calcium-sensitive probes give robust signals. These signals often are temporally resolvable in full field scans as calcium transients that persist for several seconds. Fast scanning systems, or line-scanning mode in laser scanning systems, have been used to resolve more rapid calcium events (e.g., Fan *et al.*, 1999; Wang *et al.*, 2004). Although non-ratiometric, visible wavelength calcium indicators (e.g., fluo-3, calcium green) have been more widely used in confocal applications, some studies have employed ultraviolet (UV) excited ratiometric calcium indicators, such as indo-1 (e.g., Pasti *et al.*, 2001).

In addition to calcium indicators, other fluorescent physiological probes are useful for reporting various ions including sodium, magnesium, potassium, and chloride, pH, heavy metals such as zinc, and membrane potential, to name a few (see Chapter 42, *this volume*). Although many of these probes are small molecules, genetic (GFP-based) probes have been developed (see Miyawaki, 2003) and are being incorporated into transgenic animals (e.g., Hasan *et al.*, 2004). In combination with state-of-the-art confocal and MP imaging systems, these probes will increasingly permit detailed spatio-temporal analyses of physiological processes in intact tissues and organisms (Ashworth, 2004).

Combining Fluorescence and Other Imaging Modalities

Although advancements in fluorescence imaging technology coupled with the availability of a multitude of vital fluorescent probes have combined to make fluorescence the method of choice for most high resolution studies of living cells, it is sometimes advantageous to combine fluorescence imaging with other imaging modalities. For example, differential interference contrast (DIC) microscopy can be used in conjunction with laser-scanning confocal microscopy to simultaneously monitor the whole cell in DIC mode while imaging the phagocytic uptake of fluorescent microspheres (Hook and Odeyvale, 1989) or the distribution of fluores-

TABLE 19.1. Overview of Live Cell Fluorescence Confocal Imaging Techniques

Technique	Description	Review Article(s)	Selected Examples/References
1 Time-lapse fluorescence imaging.	Repeated imaging of a field of view (single optical section) in live specimen over time.	• Cooper *et al.*, 1999	• Imaged dynamic changes in fluorescently labeled Golgi membranes (Cooper *et al.*, 1990)
2 Multi-channel or ratiometric time-lapse fluorescence imaging.	Simultaneous or sequential imaging in two or more fluorescent channels over time.	• Stricker, 2004 • Ellenberg *et al.*, 1999	• Monitored sorting of CFP- and YFP-tagged proteins through the Golgi Apparatus (Keller *et al.*, 2001)
3 Three-dimensional time-lapse (4D) imaging.	Repeated collection of *z*-series stacks of images over time.	• Gerlich & Ellenberg, 2003 • Bement *et al.*, 2003 • Hammond & Glick, 2000 • Thomas & White, 1998	• Imaged neuronal dendritic spines in brain slice cultures (Marrs *et al.*, 2001) • Imaged mitosis and migration of developing cortical neurons (Noctor *et al.*, 2004)
4 Three-dimensional multi-channel (5D) time-lapse fluorescence imaging.	Repeated collection of *z*-stacks in two or more fluorescent channels over time.	• Andrews *et al.*, 2002 • Gerlich *et al.*, 2001	• Imaged T-cell–dendritic cell interactions in lymph nodes (Stoll *et al.*, 2002) • Microglial phagocytosis in brain slices (Petersen & Dailey, 2004)
5 Spectral imaging and linear unmixing.	Method for discriminating distinct fluorophores with strongly overlapping emission spectra.	• Berg, 2004 • Zimmermann *et al.*, 2003 • Seyfried *et al.*, 2003 • Hiraoka *et al.*, 2002 • Dickenson *et al.*, 2001	• Unmix spectrally similar fluorophores in plant cells (Berg, 2004) • Resolve multiple fluorescent proteins in vertebrate cells by multiphoton imaging spectroscopy (Lansford *et al.*, 2001)
6 Fluorescence recovery after photobleaching (FRAP).	Measures recovery of fluorescence after bleaching of a portion of the specimen. Recovery may be due to protein diffusion, binding/dissociation or transport processes.	• Lippincott-Schwarz *et al.*, 2003 • Meyvis *et al.*, 1999	• Used FRAP to study integrin turnover at focal adhesions (Ballestrem *et al.*, 2001)
7 Fluorescence loss in photobleaching (FLIP).	Repeated photobleaching used to determine continuity of cell compartments and mobility of fluorescent proteins within these compartments.	• Lippincott-Schwartz *et al.*, 2001	• Dynamics and retention of correctly folded and misfolded proteins were compared in native ER membranes (Nehls *et al.*, 2000)
8 Fluorescence localization after photobleaching (FLAP).	Method for localized photo-labeling and subsequent tracking of specific molecules bearing two different fluorophores within living cells.	• Dunn *et al.*, 2002	• Used FLAP to show that actin is rapidly delivered to the leading edge of protruding cells (Zicha *et al.*, 2003)
9 Fluorescence resonance energy transfer (FRET).	Non-radiative energy transfer from a donor to an acceptor fluorophore with overlapping emission and excitation spectra. Useful for measuring interactions between two fluorescently tagged proteins.	• Sekar and Periasamy, 2003 • Wouters *et al.*, 2001	• FRET used to study activation of small G proteins during phagocytosis (Hoppe and Swanson, 2004) • FRET analysis shows that GTP-Rac coupling to effectors is locally enhanced in lamellipodia (Del Pozo *et al.*, 2002)

10	Fluorescence lifetime imaging (FLIM).	Method to investigate molecular interactions, metabolic reactions, and energy transfer in cells and subcellular structures.	• Peter and Ameer-Beg, 2004 • Periasamy *et al.*, 2002 • Bastiaens and Squire, 1999 • Pepperkok *et al.*, 1999	• Used FLIM to study interaction between CD44 and ezrin (Legg *et al.*, 2002) • Quantified dimerization of transcription factor CAATT/enhancer binding protein alpha in living pituitary cells (Elangovan *et al.*, 2002)
11	Fluorescence correlation spectroscopy (FCS).	Measures spontaneous fluorescence intensity fluctuations in a microscopic detection volume. Provides information on equilibrium concentrations, reaction kinetics, and diffusion rates of molecules.	• Bacia & Schwille, 2003 • Hess *et al.*, 2002 • Elson, 2001	• Compared mobility and molecular interactions between CaM and CaMKII in solution and in living cells (Kim *et al.*, 2004)
12	Fluorescence speckle microscopy.	Uses very low concentration of fluorescent subunits to reduce out-of-focus fluorescence and improve visibility of fluorescently labeled structures and their dynamics in thick regions of living cells.	• Adams *et al.*, 2003 • Waterman-Storer *et al.*, 1998	• Studied coupling of microtubule and actin movements in migrating cells (Salmon *et al.*, 2002)
13	Photo-uncaging/ Photoactivation.	Photo-induced activation of an inert molecule to an active state (e.g., release of a caging group from a "caged" compound), or activation of a photoactivatable fluorescent protein (e.g., PA-GFP, Kaede).	• Patterson & Lippincott-Schwartz, 2004 • Park *et al.*, 2002 • Dorman and Prestwich, 2000 • Politz, 1999	• Photo-release of caged Ca^{2+} in brain astrocytes regulates vascular constriction (Mulligan and MacVicar, 2004) • Used a reversible photoactivatable fluorescent protein to study nuclear import and export of ERK1 and importin (Ando *et al.*, 2004)
14	Optical tweezers/laser trapping.	Uses the "radiation pressure" of a stream of photons emitted from an infrared laser to "trap" small objects and molecules.	• Kuo, 2001 • Schwarzbauer, 1997	• Studied strength of cadherin adhesions in endothelial cells (Baumgartner *et al.*, 2003)
15	Fast physiological imaging • Full field • Line-scanning	Rapid, repeated collection of single scan lines or 2D images of specimen labeled with physiological indicators.	• Rubart, 2004 • Wang *et al.*, 2004 • Ashworth, 2004 • Niggli & Egger, 2004 • Miyawaki, 2003	• Imaged Ca^{2+} sparks in muscle fibers (Hollingworth *et al.*, 2000; Brum *et al.*, 2000) • Ca^{2+} imaging in neuronal dendritic spines (Pologruto *et al.*, 2004)
16	Combined fluorescence and transmitted light imaging.	Repeated simultaneous collection of one or more fluorescent channels and a transmitted light channel (e.g., DIC).	• Cogswell & Sheppard, 1991, 1992	• Imaged chromatin dynamics during the formation of the interphase nucleus (Manders *et al.*, 2003) • Imaged E-cadherin-GFP accumulation at cell adhesions in epithelial cells (Adams *et al.*, 1998).

cently tagged proteins and molecules (e.g., Adams *et al.*, 1998) within these cells. Although it is difficult to perform DIC and epi-fluorescence imaging both simultaneously and optimally in widefield microscopy, it is somewhat easier to ensure that the fluorescence signal is not subjected to the light loss that occurs in the analyzer used as part of the DIC system if one uses a single-beam confocal. Thus, the DIC image can be collected from a fluorescently labeled specimen using transmitted light that would otherwise be wasted. Recently, differential phase contrast (DPC) has been implemented in a scanning laser microscope system (Amos *et al.*, 2003), and this may offer additional capabilities where DIC optics are unsuitable. Notably for live-cell imaging, DPC reportedly needs 20 times less laser power at the specimen than DIC.

GENERAL CONSIDERATIONS FOR CONFOCAL MICROSCOPY OF LIVING CELLS

What factors must be considered when performing a live-cell confocal imaging experiment or observation? The major factors are to (1) label the preparation in order to clearly visualize the biological component of interest, (2) maintain the preparation in a condition that will support normal cell or tissue health, and (3) image the specimen with sufficient spatial and temporal resolution in a way that does not perturb or compromise it. Table 19.2 outlines several of the most important experimental considerations for live-cell imaging, including the most common problems and some potential solutions.

TABLE 19.2. Experimental Considerations for Live Cell Imaging

Consideration	Problem	Potential solution(s)
1 Temperature	Many biological phenomena are temperature sensitive.	• Use stage heaters; inline perfusion heaters; objective lens heaters; environmental boxes. • Take precautions against stage drift: — increase thermal mass, — use open-loop controls
2 Oxygenation	Most live biological specimens require O_2 (and removal of CO_2) to remain healthy. Oxygen may become depleted in closed chambers.	• Use a perfusion chamber. • Exchange used chamber media with oxygenated media intermittently or continuously. • Increase volume of chamber to promote health.
3 pH	Metabolism of live biological tissues can induce severe pH changes in chamber media over time.	• Monitor chamber pH. • Use HEPES (10–25 mM)-buffered media. • Exchange chamber media intermittently or continuously (perfusion). • Use media without phenol red pH indicator.
4 Humidity	Stage heating (especially with forced air) may cause evaporation from an open chamber, leading to dramatic changes in salinity and pH.	• Use closed chamber configuration (perfusion chamber). • Use humidified environmental box. • Use auto-fill system for open chambers.
5 Fluorescence signal strength	Weakly fluorescent probes or low concentration of probes can yield weak signals that produce images with low signal-to-noise ratio.	• Increase pixel dwell time. • Open confocal pinhole aperture (e.g., to >2 Airy disks). • Maximize throughput of emission pathway (e.g., in spectral imaging systems with variable spectral filters). • Use line or frame averaging to improve signal-to-noise ratio. • Adjust illumination (filling) of back aperture of objective lens.
6 Channel bleed-through or cross-talk	In biological specimens labeled with multiple fluorescent probes, signals from one channel may be detected in other channels.	• Image separate fluorescence channels sequentially (either line-by-line or frame-by-frame in scanning systems). • Use spectral imaging and linear unmixing algorithms. • Use modern, hard-coated interference filters and dichroics.
7 Photobleaching	Fluorescent probes bleach with repeated illumination. Some fluorescent probes bleach quickly.	• Reduce incident illumination. Then reduce it again! • Use fade resistant dyes. • Open confocal pinhole aperture. • Maximize throughput of emission pathway (e.g., in spectral imaging systems with variable spectral filters). • Reduce pixel dwell time (in scanning systems). • Reduce frequency of image capture. • Blank laser beam during flyback (in scanning systems). • Only scan specimen when actually collecting data. • To improve S/N, always deconvolve 3D data before viewing.
8 Spatial resolution	Some observations require very high spatial resolution in *x*-*y* or *z*.	• Use high NA objectives. • Reduce size of confocal pinhole aperture (to ~1 Airy disk). • Increase spatial sampling frequency (guided by Nyquist theorem). • Increase electronic zoom (but avoid empty magnification). • Decrease step size in *z*-stacks. • Use water-immersion objective lenses to reduce spherical aberration. • Deconvolve the images.
9 Temporal resolution	Some biological phenomena are rapid relative to the rate of image collection (especially problematic with laser scanning confocal systems).	• Reduce field of view (e.g., collect fewer horizontal lines). • Reduce pixel dwell time (e.g., increase scan speed). • Reduce spatial sampling frequency (e.g., reduce pixel array from 1024 to 512).
10 Focus drift	Live biological specimens on heated microscope stages, or features within live specimens (e.g., mitotic cells), can move relative to a fixed focal plane.	• Collect a *z*-stack of images, and reconstruct these images following the observation. • Manual focus adjustments may be required periodically. • Auto-focus methodology may be employed in some cases.

Maintenance of Living Cells and Tissue Preparations

In Vitro Preparations

Specimen maintenance is a very important part of any live imaging study and usually requires both mechanical ingenuity and insight into the biology of the cell or tissue under study. The specimen chamber must keep the cells or tissues healthy and functioning normally for the duration of the experiment while allowing access to the microscope objective. This can be particularly difficult when high-numerical-aperture (NA) oil- or water-immersion lenses are used. In many cases, there must also be a controlled and efficient way to introduce a reagent to perturb a particular cellular process. Other important factors are simplicity, reliability, and low cost. It is advisable to monitor the conditions within the imaging chamber carefully. It may be helpful to use microprobes that can detect pH, O_2, and CO_2 (e.g., Lazar Research Laboratories, Inc., Los Angeles, CA).

The early closed perfusion chambers designed by Dvorak and Stotler (1971) and later by Vesely and colleagues (1982) were inexpensive and permitted high-resolution transmitted light observation. They relied on an external heater that warmed the entire stage area for temperature control.

Setups for different cells vary widely. Mammalian cells probably pose the greatest problems. McKenna and Wang's article (1986) is a general introduction to the problems associated with keeping such cells alive and functioning on the microscope stage. This article discusses culture chamber design as well as strategies for controlling pH, osmolarity, and temperature. The authors describe their own chamber, in which temperature is controlled by heating the air in a box surrounding the stage area, and mention earlier designs such as the resistively heated Lieden Culture System first described by Ince and colleagues (1983) and later improved by Forsythe (1991).

Strange and Spring (1986) describe their setup for imaging renal tubule cells where temperature, pH, and CO_2 are controlled. They provide a detailed account of the problems of establishing laminar flow perfusion systems, temperature regulation, and maintenance of pH by CO_2 buffering. Somewhat later, Delbridge and colleagues (1990) describe a sophisticated, open-chamber superfusion system permitting programmed changes of media, precision control of media surface height, and temperature regulation between 4°C and 70°C using a Peltier device to control the perfusate temperature. Myrdal and Foster (1994) used a temperature-stabilized liquid passing through a small coil suspended in media filling a plastic Nunc chamber to provide temperature control for confocal observations of the penetration of fluorescent antibodies into solid tumor spheroids. An automatic system maintained fluid level and bathed the area in CO_2 but special precautions were required to prevent drift of the confocal focus plane during long time-lapse sequences. Methods for observing microglial cell movements in mammalian brain slices are described in detail in a later section of this chapter.

Chambers have even been built for the microscopic observation of cells as they are being either frozen or thawed in the presence of media that could be changed during the process (e.g., Walcerz and Diller, 1991). In this case, computer-controlled pumps deliver temperature-controlled nitrogen gas at between −120°C and 100°C to special ports connected to a temperature cell (−55°C to 60°C) that forms the upper boundary of the perfusion chamber. Other ports carry either the perfusate or a separate nucleating agent to the cell chamber itself.

More recently, a specialized *in vitro* cell culture system has been developed to maintain mammalian neuronal cells for over a year (Potter and DeMarse, 2001)!

There are several companies that provide ready-made microscope stage chambers, temperature-control units, automated perfusion systems, and a variety of related accessories. These are summarized in Table 19.3.

In Vivo Preparations

The ultimate goal of many research programs is to understand the normal (or abnormal) structure and function of molecules, cells, and tissues *in vivo*, that is, in the living organism functioning within its native environment (Frosting, 2002; Megason and Fraser, 2003). There has been some remarkable progress recently on extending high resolution confocal and MP imaging in this direction, especially in preparations that are essentially translucent. Several model organisms, including zebrafish (Cooper *et al.*, 1999), frog (Fraser and O'Rourke, 1990; Robb and Wylie, 1999), fruit fly (Paddock, 2002), leech (Baker *et al.*, 2003), and worm (Crittenden and Kimble, 1999), have emerged as excellent preparations for cellular and molecular imaging studies spanning a variety of biological questions. As an example, studies in the zebrafish have been carried out on the structural development of vasculature (Lawson and Weinstein, 2002; Isogai *et al.*, 2003), cell division (Gong *et al.*, 2004; Das *et al.*, 2003), neuronal migration (Koster and Fraser, 2001), axonal pathfinding (Dynes and Ngai, 1998), synapse formation (Jontes *et al.*, 2000; Niell *et al.*, 2004), and synaptic plasticity (Gleason *et al.*, 2003), to name a few. Physiological studies in zebrafish have included, for example, imaging intracellular calcium during gastrulation (Gilland *et al.*, 1999), in the intact spinal cord (O'Malley *et al.*, 1996; Gahtan *et al.*, 2002), and in brain (Brustein *et al.*, 2003). Other examples related to studies of embryos are covered in Chapter 43. Each of these biological preparations embodies its own unique set of specimen mounting and maintenance challenges. Indeed, it is sometimes necessary to anesthetize the preparation to prevent it from crawling or swimming away during the imaging session!

Perhaps the most difficult conditions involve imaging in a living mammal, an undertaking for which the confocal or MP microscope enjoys the twin advantages of epi-illumination and optical sectioning that make it possible to view solid tissues without mechanical disruption. Confocal microscopy has long been an important tool for *in vivo* imaging of eye tissues non-invasively (Petran *et al.*, 1986; Jester *et al.*, 1991, 1992; Masters, 1992; Petroll *et al.*, 1992, 1993; Poole *et al.*, 1993). In terms of imaging interior tissues, early studies described methods for examining microcirculation of the brain cortex in anesthetized rats (Dirnagl *et al.*, 1992) or changes in kidney tubules during ischemia (Andrews *et al.*, 1991). Confocal microscopy also has been used to image leukocyte–endothelium interactions during infections through closed cranial windows (Lorenzl *et al.*, 1993). More recently, MP has been used to image live mammalian brain tissues *in vivo*, either through a cranial window (Svoboda *et al.*, 1997; Trachtenberg *et al.*, 2002), fiber-optic coupled devices (Mehta *et al.*, 2004), or directly through the intact but thinned skull (Yoder and Kleinfeld, 2002; Zhang *et al.*, 2005). Dual-channel MP imaging also has been used to image other tissues *in vivo*, including lymphoid organs (e.g., Miller *et al.*, 2002). It is generally accepted that MP imaging is superior to single-photon confocal for these *in vivo* imaging studies (Cahalan *et al.*, 2002).

Fluorescent Probes

Except in those cases where an adequate image can be derived from either the backscattered-light signal or from autofluorescence, confocal microscopy of living cells is dependent on the properties and availability of suitable fluorescent probes. In addition to binding specifically to what one is interested in studying,

TABLE 19.3. Commercially Available Chambers for Live Cell Imaging

Source	Description/Features	Contact Info
20/20 Technology, Inc. Bldg. 2, Unit A 311 Judges Road Wilmington, NC 28405 USA	Heating, cooling, atmosphere control instrumentation for microscopy.	TEL: 1-910-791-9226 WEB: http://20-20tech.com/
ALA Scientific Instruments Inc. 1100 Shames Dr. Westbury, NY 11590 USA	Microincubators and temperature control; Peltier heating & cooling pre-stage; recording chambers; inline perfusion heating tube.	TEL: 516-997-5780 WEB: www.alascience.com EMAIL: sales@alascience.com
ASI / Applied Scientific Instrumentation Inc. 29391 W. Enid Rd. Eugene, OR 97402 USA	Supplier for Solent and Bioptechs incubation chambers.	TEL: 541-461-8181 WEB: http://www.asiimaging.com/ EMAIL: info@ASIimaging.com
AutoMate Scientific, Inc. 336 Baden Street San Francisco, California 94131 USA	Programmable controlled perfusion systems, temperature control, valves and fittings, oocyte perfusion chamber, Petri dish perfusion chamber, sub-millisecond switching, submerged and interface tissue and brain slice chambers.	TEL: 415-239-6080 WEB: http://www.autom8.com/ EMAIL: info@autom8.com
Bellco Glass, Inc. 340 Edrudo Road, Vineland, NJ 08360 USA	Sykes-Moore culture chambers; used with stationary culture when medium is changed intermittently.	TEL: 1-800-257-7043 WEB: http://www.bellcoglass.com/ EMAIL: cservice@bellcoglass.com
BioCrystal Ltd OptiCell 575 McCorkle Blvd. Westerville, OH 43082 USA	OptiCell is a sterile, sealed cell culture environment between two optically clear gas-permeable growth surfaces in a standard microtiter plate-sized plastic frame with ports for access to the contents.	TEL: 614-818-0019 WEB: http://www.opticell.com EMAIL: sales@opticell.com
Bioptechs, Inc. 3560 Beck Road Butler, PA 16002 USA	Live cell microscopy environmental control systems. Thermal regulation of specimen and objective, electronic control and integration of temperature and perfusion.	TEL: 724-282-7145 WEB: http://www.bioptechs.com/ EMAIL: info@bioptechs.com
Bioscience Tools — CB Consulting Inc., 4527 52nd Street, San Diego, CA 92115 USA	Glass bottom Petri dishes; ultra-thin imaging chambers; temperature control; perfusion systems; small volume delivery systems; ultra-fast temperature/solution switch.	TEL: 1-877-853-9755 WEB: http://biosciencetools.com/ EMAIL: info@biosciencetools.com
C&L Instruments, Inc. 314 Scout Lane Hummelstown, PA 17036 USA	Fluorometers and fluorometer components for steady-state fluorescence measurements; complete fluorescence systems for photometry and fluorescence imaging.	TEL: 1-717-564-9491 WEB: http://www.fluorescence.com/ EMAIL: support@fluorescence.com
CellBiology Trading Hinsbeker Berg 28a Hamburg, 22399 Germany	Microinjection and incubation; EMBL live cell observation chamber.	TEL: 49-0-40-53889432 WEB: http://cellbiology-trading.com/ EMAIL: info@cellbiology-trading.com
Dagan Corporation 2855 Park Avenue, Minneapolis, Minnesota 55407 USA	Microscope stage temperature controller; perfusion controller.	TEL: 612-827-5959 WEB: http://www.dagan.com/ EMAIL: support@dagan.com
Digitimer Ltd 37 Hydeway Welwyn Garden City Hertfordshire, AL7 3BE, England	AutoMate Scientific, Medical Systems and Scientific Systems Design incubators, chambers, and perfusion systems.	TEL: +44 (0) 1707 328347 WEB: http://www.digitimer.com/ EMAIL: sales@digitimer.com
Grace Bio-Labs, Inc. P.O. Box 228 Bend, OR 97709 USA	Manufactures 3-D microporous coatings on microscope slides, and a variety of "press to seal" enclosures for microarrays, cell culture, and high throughput cytochemistry, hybridization, cytogenetics, and fluorescent imaging applications.	TEL: 1-800-813-7339 WEB: http://www.gracebio.com/ EMAIL: custservice@gracebio.com
Harvard Apparatus Inc. 84 October Hill Rd. Holliston, MA 01746 USA	Variety of valve controlled perfusion systems.	TEL: 508-893-8999 WEB: http://www.harvardapparatus.com EMAIL: bioscience@harvardapparatus.com
Integrated BioDiagnostics Schellingstrasse 4 80799 München, Germany	The μ-slide family of live cell imaging flow chambers; suited for optical studies of hydrodynamic shear stress on biofilms or adhesion studies on cell layers.	TEL: +49 (0)89 / 2180 64 19 WEB: http://www.ibidi.de/ EMAIL: info@ibidi.de
Intracel, Ltd. Unit 4 Station Road Shepreth, Royston Herts, SG8 6PZ England	WillCo glass bottomed dishes; Bioptechs micro-environmental control systems.	TEL: 01763 262680 WEB: http://www.intracel.co.uk/ EMAIL: intracel@intracel.co.uk
In Vitro Systems & Services GmbH Rudolf-Wissell-Str. 28 37079 Göttingen, Germany	Gas-permeable plastic foil (bioFOLIE 25); sterile tissue culture dish (petriPERM); Petri dish with gas-permeable base; two-compartment system.	TEL: ++49 551 500 97-0 WEB: http://www.ivss.de/ EMAIL: info@ivss.de
Life Imaging Services Kaegenstrasse 17 CH-4153 Reinach, Switzerland	Ludin imaging chamber; microscope temperature control system	TEL: ++41 (0)61 7116461 WEB: http://www.lis.ch/ EMAIL: info@lis.ch
MatTek Corporation 200 Homer Avenue Ashland, MA 01721 USA	Glass bottom culture dishes	TEL: 1-800-634-9018 WEB: http://www.glass-bottom-dishes.com/ EMAIL: DishInfo@mattek.com
Molecular Probes, Inc. 29851 Willow Creek Road Eugene, OR 97402 USA	Attofluor cell chamber designed for viewing live-cell specimens on upright or inverted microscopes. Chamber gaskets for imaging, perfusion, and incubation.	TEL: 1-541-465-8300 WEB: http://www.probes.com/ EMAIL: tech@probes.com

TABLE 19.3. (*Continued*)

Source	Description/Features	Contact Info
PeCon GmbH Ziegeleistraße 50 89155 Erbach Germany	Live cell imaging solutions including stage heating and cooling, CO_2 and O_2 regulation, and evaporation reduction.	TEL: 0049 (0) 7305 95666-0 WEB: http://www.pe-con.de/pecon/index.htm EMAIL: info@pecon.biz
Physitemp Instruments, Inc. 154 Huron Avenue Clifton, New Jersey 07013 USA	Heating & cooling stages (Peltier) for microscopes (–20° to +100°C); custom thermal stages.	TEL: 1-973-779-5577 WEB: http://www.physitemp.com/ EMAIL: physitemp@aol.com
SDR Clinical Technology 213 Eastern Valley Way Middle Cove, NSW 2068 Australia	Physiological recording chambers & accessories for use on the microscope stage; bath perfusion; temperature control.	TEL: +61-2-9958-2688 WEB: http://www.sdr.com.au/ EMAIL: sdr@sdr.com.au
Solent Scientific Limited 14 Matrix Park, Talbot Road, Segensworth PO15 5AP, UK	Manufacturers of full enclosure incubation chambers for research inverted microscopes, confocal microscopes and multi-photon microscopes.	TEL: +44 (0)870 774 7140 WEB: http://www.solentsci.com/ EMAIL: sales@solentsci.com
Stratech Scientific, Ltd. Unit 4 Northfield Business Park, Northfield Road, Soham, Cambridgshire CB7 5UE UK	CoverWell imaging chambers are designed to stabilize and support thick and free-floating specimens for confocal microscopy and imaging applications.	TEL: +44 (0)1353 722500 WEB: http://www.stratech.co.uk/
Warner Instruments, Inc. 1125 Dixwell Avenue, Hamden, CT 06514, USA	Full range of recording, imaging, and perfusion chambers; perfusion and valve control.	TEL: 1-800-599-4203 WEB: http://www.warneronline.com/ EMAIL: support@warneronline.com
WillCo Wells BV WG Plein 287 1054 SE Amsterdam, The Netherlands	WillCo-dish glass bottom dishes.	TEL: ++31(0)20 685 0171 WEB: http://www.willcowells.com EMAIL: info@willcowells.com
World Precision Instruments, Inc. 175 Sarasota Center Boulevard Sarasota, Florida 34240 USA	FluoroDish glass-bottom culture dish; Air-Therm ATX Air Heater Controller; programmable automated multi-channel perfusion system.	TEL: 941-371-1003 WEB: http://www.wpiinc.com/

the fluorescent probe should produce a strong signal and be both slow to bleach and non-toxic. Chapters 16 and 17 discuss fluorescent dyes that have been used in published work with confocal microscopy in detail.

Many dyes are useful when introduced to the medium surrounding cells to be labeled. Some of the classic and most commonly used cell stains include DiI for labeling the plasma membrane (Honig and Hume, 1986; Baker and Reese, 1993), $DiOC_6(3)$ for labeling internal membranes (Terasaki *et al.*, 1984), NBD–ceramide and bodipy–ceramide which label the Golgi apparatus (Pagano *et al.*, 1991), rhodamine 123 which labels mitochondria (Johnson *et al.*, 1980), potential sensitive dyes such as $DISBAC_2(3)$ (see Fig. 8.65, *this volume*) (Loew, 1993), and FM 1-43 (Betz *et al.*, 1992) which is used to follow plasma membrane turnover and vesicular release. Fluorescent ion indicators such as Fluo-3 (Minta *et al.*, 1989) can either be microinjected or added to the media in a cell-permeant acetoxymethylester form that becomes trapped inside the cell after being cleaved by intracellular esterases (see Chapter 42, *this volume*).

Minimizing Photodynamic Damage

Once the cells are labeled and on the microscope, one is faced with the challenge of collecting data without compromising the cell or bleaching the label. In practice, the major problem is light-induced damage. Fluorescent molecules in their excited state react with molecular oxygen to produce free radicals that can then damage cellular components and compromise cell health (Dixit and Cyr, 2003; see also Chapters 38 and 39, *this volume*).

In addition, several studies suggest that components of standard culture media might also contribute to adverse light-induced effects on cultured cells (see Siegel and Pritchett, 2000). Some early studies (Spierenburg *et al.*, 1984; Zigler *et al.*, 1985, 1991; Lepe-Zuniga *et al.*, 1987) indicated a phototoxic effect of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) containing media on cells under some circumstances. It seems possible that this effect might be more directly related to inadequate levels of bicarbonate (Cechin *et al.*, 2002). Other studies suggest that riboflavin/vitamin B2 (Zigler *et al.*, 1985; Lucius *et al.*, 1998) and the essential amino acid tryptophan (Griffin *et al.*, 1981; Silva *et al.*, 1991; Silva and Godoy, 1994; Edwards *et al.*, 1994) may mediate phototoxic effects. Whether these effects occur under typical confocal imaging conditions is unknown, but many of the photoeffects are reduced by antioxidants, so it seems advisable to maintain antioxidants (and some bicarbonate, as well) in the specimen chamber (see below) and to use photons with great efficiency.

Improving Photon Efficiency

There are several strategies to minimize the amount of excitation light required to collect data (see Chapters 2 and 9, *this volume*, for more details). Briefly, higher-NA objective lenses collect more of the fluorescent emission. For a given lens, there is also a theoretical optimal setting of the zoom magnification that best matches the resolution required to the allowable dose (see Chapter 4, *this volume*). When the focus plane is more than 5 µm from the coverslip, water-immersion lenses should be used to avoid the signal loss caused by spherical aberration when using an oil lens (see Chapters 7 and 20, *this volume*).

Another way to reduce light damage is to minimize the duration of the light exposure during the experimental setup. For example, one should try to focus as rapidly as possible and turn off the light source as soon as the focus range has been chosen. In addition, in single-beam scanning systems, make sure that your scanner is set up to blank the laser beam during scan retrace. Otherwise, areas on both sides of the imaged area will receive a very high light exposure as the beam slows down to change direction.

Finally, photon efficiency can be maximized by using the best mirrors, the correct pinhole size for the resolution required (in *x*,

y, and *z*), and photodetectors that yield the highest quantum efficiency at the wavelength of the signal.

Antioxidants

As noted above, one can also reduce photodynamic damage by adding antioxidants to the medium. Oxyrase (Oxyrase Inc., http://www.oxyrase.com) is an enzyme additive used to deplete oxygen in order to grow anaerobic bacteria. It has been used at 0.3 unit/mL to reduce photodynamic damage during observations of mitosis (Waterman-Storer *et al.*, 1993). Another approach is to include ascorbic acid in the medium. This reducing agent is typically used at 0.1 to 1.0 mg/mL but has been used at up to 3 mg/mL. A recent confocal study of calcium transients in isolated chondrocytes reported a relationship between laser intensity and the frequency of Ca^{2+} oscillations and cell viability: Ca^{2+} events were more frequent and cell viability was decreased with higher laser intensity (Knight *et al.*, 2003). Treatment with ascorbic acid reduced the Ca^{2+} events and improved cell viability (see also Chapters 16 and 17, *this volume*).

THE ONLINE CONFOCAL COMMUNITY

Confocal microscopy of living cells is an area of active research where individuals are constantly developing new techniques and approaches. One way to keep up with current practice is to join about 1600 others who subscribe to the Confocal e-mail listserver. This can be done by registering at the listserver Web site, located at http://listserv.buffalo.edu/archives/confocal.html. You will then begin to receive messages from other microscopists. Recent topics have included discussions on such diverse issues as autofluorescence problems, glass-bottomed culture chambers, damage to live cells during FRAP experiments, and announcements of confocal workshops. The listserver also has an extensive, searchable archive dating back to 1991, and this is freely accessible.

A CONVENIENT TEST SPECIMEN

Knebel *et al.* (1990) showed that onion epithelium (*Allium cepa*) is a simple preparation that can be used as a convenient test specimen for confocal microscopy of living cells. Figure 19.1 shows how to prepare onion epithelium. First, a small square of a layer is cut out using a razor blade. A forceps is used to peel off the thin epithelium on the inner surface of the onion layer. The epithelium is then put onto a microscope slide, covered with a drop or two of staining solution containing $DiOC_6(3)$, a marker of mitochondria and endoplasmic reticulum, and coverslipped. The stock solution of $DiOC_6(3)$ (0.5 mg/mL in ethanol) can be kept indefinitely if protected from light in a scintillation vial. The staining solution is a 1 : 1000 dilution in water on the day of the experiment. The center of these cells is usually occupied by a large vacuole, and the ER and mitochondria are located in a thin cytoplasmic region near the plasma membrane. Motion of the ER is relatively quick and easily detected in consecutive 1-s scans.

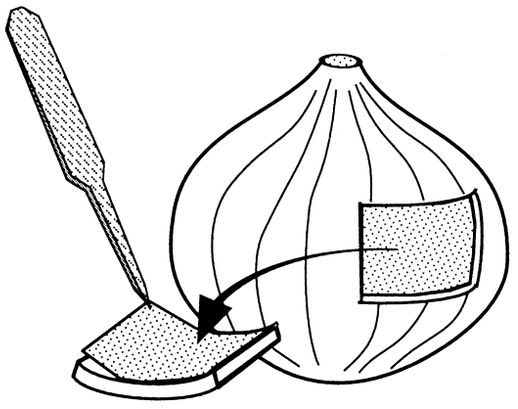

FIGURE 19.1. A convenient test specimen for confocal microscopy of cells in a living tissue: onion epithelium. This drawing shows how to obtain onion epithelium. As described in the text, the epithelium is stained with $DiOC_6(3)$, and the ER and mitochondria within the cells provide a bright and motile specimen.

SPECIFIC EXAMPLE I: VISUALIZING CHROMATIN DYNAMICS USING VERY LOW LIGHT LEVELS

It is clear from the discussion above that microscopy of living cells has become a technique of major importance in cell biology: it can be used to tell us *where* molecules are located, *when* they become localized, *how fast* they are moving, with which molecules they are *interacting*, and how long they stay *attached* to these molecules. All these properties can be observed in the natural environment of the living cell. The major limiting factor in live-cell imaging is phototoxic effect of light used for the observation of the cell. Here we will address some practical issues of phototoxicity based on our experience in imaging chromatin dynamics in living cells (Manders *et al.*, 1996; 1999; 2003; Verschure *et al.*, 1999; Moné *et al.*, 2004).

Phototoxicity

A large number of photochemical reactions are responsible for the phototoxic effect of light. Light can be absorbed by cellular components and induce chemical alterations in their molecular structure. For example, UV light is absorbed by DNA (absorption peak at 280 nm), directly inducing DNA damage. Here we assume that, working with visible light, the direct photodamage is negligible. In fluorescently labeled cells, the main source of photodamage is the production of reactive oxygen species (ROS) including singlet oxygen ($^1O_2$), superoxide ($\cdot O_2^-$), hydroxyl radical (HO·), and various peroxides. These activated oxygen species react with a large variety of easily oxidizable cellular components, such as proteins, nucleic acids, and membrane lipids. Singlet oxygen is responsible for much of the physiological damage caused by reactive oxygen species. For the production of singlet oxygen, the fluorescent label acts as a photosynthesizer in a photochemical reaction where dioxygen ($^3O_2$) converts into singlet oxygen ($^1O_2$). Singlet oxygen mainly modifies nucleic acid through the selective oxidation of deoxyguanosine into 8-oxo-7,8-dihydro-2′-deoxyguanonine. Proteins and lipids also will be damaged by ROS. Phototoxicity likely depends on several variables:

- **The photochemical properties of the fluorescent molecule.** Some molecules induce more phototoxicity than others, depending on the lifetime of their triplet state. For photodynamic therapy (PDT), dedicated molecules called photosensitizers have been designed in order to induce a maximum damage in tissue for the treatment of cancer (e.g., halogenated fluorescein is much more toxic than fluorescein). Another property that influences the phototoxicity of a molecule is the

local environment of the molecule. The active fluorophore of a GFP molecule is positioned on the inside of the protein, within the barrel structure (the "β-can"). Probably this hydrophobic protein environment contributes to the relatively low phototoxicity of GFP compared with naked fluorophores such as fluorescein or rhodamine.

- **The subcellular location of the fluorescent molecule.** When fluorescent molecules are situated close to DNA, the damaging effect of singlet oxygen is more pronounced. Despite several DNA-repair mechanisms, the cell will not continue its cell cycle (arrest) and may even die if there is too much DNA damage. Therefore, fluorophores in the cytoplasm seem to induce less phototoxicity than fluorophores in the nucleus.
- **The concentration of fluorophore.** It is clear that there is a relationship between the local concentration of fluorophore and the level of phototoxicity. We assume a linear relationship between fluorophore concentration and toxicity, although this has not been assessed directly and is complicated by the fact that if there is more dye, one need use less excitation.
- **The excitation intensity.** Fluorescent cells in a dark incubator are quite happy for weeks as long they are maintained in the dark. As the word *phototoxicity* implies, photons are needed to induce toxicity in a fluorescently labeled specimen. We usually assume a linear relationship between excitation light dose and toxicity, although the temporal regimen of the excitation may be important to how cells handle the accumulation of phototoxic biproducts. Phototoxicity is dependent on the wavelength of light in the sense that the wavelength of the toxic excitation light matches the excitation curve of the fluorophore. In other words, it is the excited fluorophore that is toxic. Koenig also found that, with two-photon excitation, the damage is proportional to the number of molecular excitations (see Chapter 38, *this volume*).

There is no clear evidence for differences in phototoxicity between green, red, or far-red fluorophores. In principle, excited Cy5 can be as toxic as excited FITC. However, the wavelength of excitation light can be a factor when imaging in thick specimen because stronger incident illumination is needed for comparable excitation of shorter wavelength fluorophores due to increased tissue scatter at shorter wavelengths.

Reduction of Phototoxicity

For many researchers, phototoxicity is a serious (and annoying!) limitation of their observations of living cells. When you do not look at a cell it is alive, but the moment you start to observe how it lives, it is killed by the light used to observe it. In experiments so far, we have succeeded in obtaining acceptable time series of living cells by carefully optimizing all steps in the imaging process in an effort to achieve (i) maximal signal-to-noise ratio (S/N), (ii) maximal spatial and temporal resolution, and (iii) minimal phototoxic effects. Specifically, phototoxicity has been minimized by (i) using radical scavengers (e.g., trolox) in the culture medium and (ii) using culture medium without phenol-red. Most important of all, however, is minimizing the total excitation light dose. The excitation light dose is the product of the light intensity and the exposure time. Decreasing either the excitation intensity or the excitation dose implies a loss of fluorescent signal. It is inevitable that a reduction of light dose puts a limitation on the S/N and the spatial and temporal resolution. The art of successful live-cell microscopy is finding the balance between image quality and cell vitality.

Improving Image Quality in Low-Dose Microscopy

Figure 19.2(A) shows a single time frame projection from a 3D time-series of a HeLa cell expressing the fluorescent histone fusion protein, H2B-GFP. In the time series shown in Figure 19.2(B), the cell is in late telophase at the start of the imaging and proceeds into interphase during the movie. This movie shows data from a study on the dynamics of chromatin during decondensation (Manders *et al.*, 2003).[1] In these experiments, the excitation light intensity was kept below 150 nW and the total exposure time[2] of a cell that was 3D imaged for 3 h was not more than 70 s. Under these conditions the total light dose was approximately $10\,J\,cm^{-2}$. In experiments where we used a higher dose of light we observed phototoxic effects, such as cell cycle arrest and cell death.

Reducing the total light dose during an experiment requires that the number of 3D images in the sequence (temporal sampling rate) be low. Because of this limited sampling rate, live-cell movies are usually under-sampled in time according to the Nyquist criterion. As a result, such movies often show cells that nervously move from one place to another and sometimes suddenly rotate. We have applied an image processing procedure to correct for all the movements (translation and rotation) of the cell. For each 3D image of the time sequence, a translation and rotation transform vector was calculated in order to obtain a best fit with the previous image in the sequence. After a series of such transformations, a new movie was produced showing a stable cell that does not move or rotate. Only internal movements are visible. After this correction procedure, we applied a simple Gaussian spatial filter to reduce noise in the image [Fig. 19.2(C,D)]. We also applied a temporal filter by adding to each voxel of the 3D image at each time-point the value for that voxel in the previous and subsequent image multiplied by an intensity factor of 0.5. Our experience is that temporal filtering makes the movie easier to interpret.

Low-Dose Imaging Conclusion

The success of live-cell microscopy is very much dependent on minimizing or avoiding any toxic effect of light on the biological system under observation. A certain dose of light may induce serious DNA damage that may arrest the cell cycle, whereas the diffusion coefficient of a certain protein is not influenced at all at the same dose. In the experimental example shown here [Fig. 19.2(B)], we used only 150 nW of incident beam power. This dose was found to be phototoxic in other experiments using fluorescein instead of GFP, and it was found necessary to drop the laser power to 50 nW [Fig. 19.2(E)]. These power levels are far lower than (i.e., <1% of) those commonly used in confocal microscopy, a circumstance facilitated at least in part by the fact that the chromosomes are quite heavily stained.

Our collective experiences indicate that the effect of phototoxicity depends on the cell type, the stage of the cell cycle, the fluorophore, the observed biological process, and many other experimental conditions. We conclude that there is no general guideline for the maximum allowable laser power: it must be assessed empirically for each experimental condition. As a general

[1] Both movies are on the Springer Web site associated with this book.
[2] Power levels were measured using a photometer sensor that was oil-coupled to the specimen side of an oil-coupled coverslip.

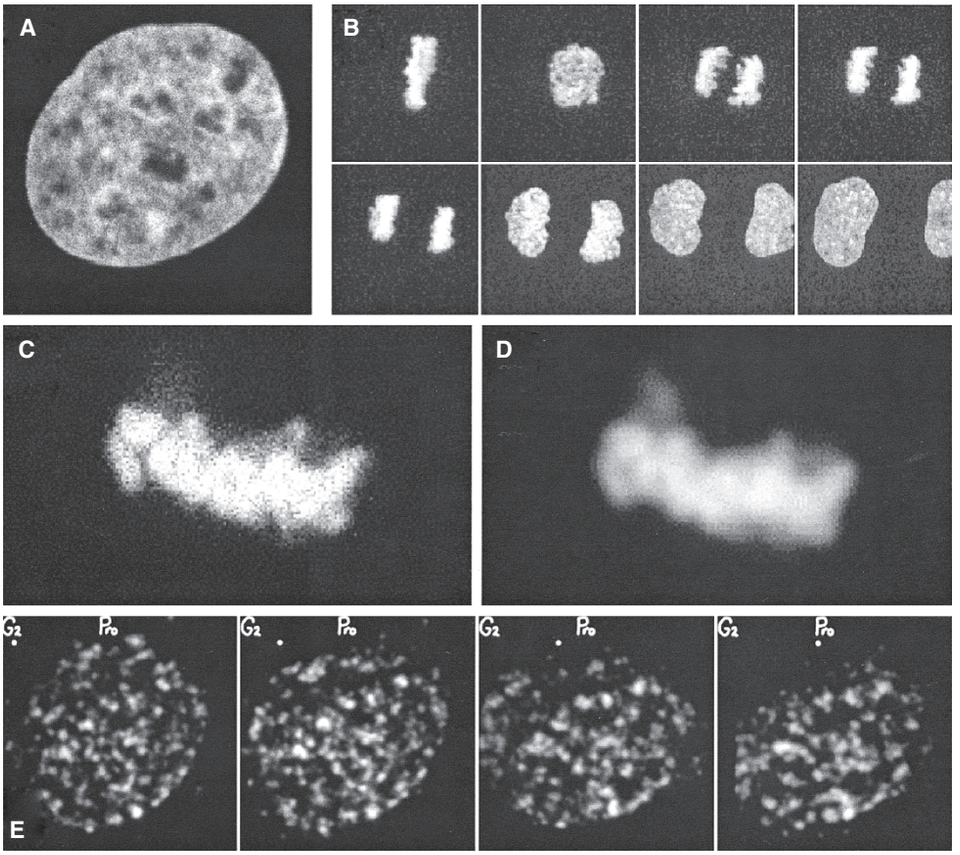

FIGURE 19.2. (A) A single time frame from a time-series of a HeLa cell expressing the fluorescent histone fusion protein, H2B-GFP. The cell is in late telophase. (B) A time-series of another cell shows how it proceeds into interphase during chromatin decondensation. In this experiment, the excitation light intensity was kept below 150 nW. Higher laser power induced cell cycle arrest. (C, D) Application of a simple Gaussian spatial filter reduces noise in the raw image (C) and improves image quality (D). (E) Time-series showing chromatin structure in a cell proceeding from G2 (*left*) to prophase (*right*). Note that the images were captured with a very low power dose (50 nW). See the supplemental video movie at http://www.springer.com/0-387-25921-X. For details, see Manders and colleagues (2003).

rule, however, images of living cells are almost always more noisy than images of fixed preparations because the incident illumination intensity needs to be kept to a minimum to maintain cell viability. A noisy image in which one can see what is absolutely essential is of more use than a "better" image of a damaged cell. Keep in mind that not all types of damage are equally easy to detect. Damage may disturb (or create!) a monitored process, it may interfere with cell division, or it may cause the cell to bleb and pop! We recommend that the experimentalist BEGIN by assuming that "to observe is to disturb." Measure the power level coming out of the objective. Try your experiment again using twice the power, and again using half the power. Make sure that you can explain any "behavioral" differences between these runs.

We show here that some simple image processing (deconvolution) can help facilitate the analysis of live-cell imaging data by reducing noise and improving the point-to-point coherence when viewing a time-series of images. Note the reduction in noise level between Figure 19.2(C) and 19.2(D).

SPECIFIC EXAMPLE II: MULTI-DIMENSIONAL IMAGING OF MICROGLIAL CELL BEHAVIORS IN LIVE RODENT BRAIN SLICES

The example above serves to illustrate that confocal microscopy is an important tool for studying dynamic subcellular processes in live, isolated cells. Many biologists are also interested in understanding dynamic structural and functional aspects of cells within the context of a natural tissue environment. As noted above, confocal and multi-photon imaging have been applied to intact, normally functioning systems such as the eye, skin, or kidney. Some recent studies have even extended these observations beyond superficial tissues to deep tissues of the brain (e.g., Mizrahi *et al.*, 2004).

However, some tissues are much less accessible, or it may be of interest to be able to experimentally perturb or control the system under study. For these purposes, the *in vitro* tissue slice has been an important experimental preparation (e.g., Gähwiler *et al.*, 1997). Smith and colleagues (1990) were among the first to show the feasibility of imaging the structure and physiology of living mammalian brain tissue slices at high resolution using fluorescence confocal microscopy. Since then many confocal studies of both the structural and the physiological dynamics of cells in tissue slices have appeared, and it seems that interest in imaging *in vitro* tissues is continuing to grow. We will address here some of the most common problems, challenges, and limitations inherent in confocal studies of live tissue slices. These points will be illustrated by drawing from our own time-lapse studies in live tissue slices of developing mammalian central nervous system (CNS) (Dailey and Smith, 1993, 1994, 1996; Dailey *et al.*, 1994; Dailey and Waite, 1999; Marrs *et al.*, 2001; Stence *et al.*, 2001; Grossmann *et al.*, 2002; Petersen and Dailey, 2004; Benediktsson *et al.*, 2005).

Some of the major problems encountered when imaging fluorescently labeled cells in live tissue slices are:

- **Attaining a suitable level and specificity of staining.**
- **Maintaining cell/tissue health**: pH, temperature, oxygen, etc.

- **Keeping cells in focus**: Can be an immense problem when following cells over long periods of time:
  - Movement of the microscope stage, especially when stage heaters are used.
  - Movement of the tissue: apparent movement that is really caused by movement of the focal plane within the specimen; natural movement of whole organisms or those caused by heartbeat, etc.
  - Movement of cells within the tissue, for example, cell migration, extension/retraction of cell processes.
  - Movement related to experimental procedures, for example, stimulus-induced osmotic changes.
- **Attaining a useful image with a high S/N of cells deep within tissue:**
  - Imaging away from damaged tissue surfaces.
  - Light scatter by the tissue.
  - The problem of spherical aberration.
- **Handling data**: viewing, storing, retrieving, and analyzing four-dimensional (4D) data sets:
  - Short term: monitoring experiments on the fly; adjusting focus.
  - Long term: accessibility and security of archived data.

We have been exploring the dynamic behavior of a type of brain cell, termed microglia, following brain tissue injury. These cells undergo a dramatic transformation ("activation") from a resting, ramified form to an amoeboid-like form within a few hours after traumatic tissue injury. Activation of microglia is triggered by signals from injured cells (including neurons), and this mobilizes microglia to engage neighboring dead and dying cells. Naturally, these events are best studied in the context of a complex tissue environment containing the native arrangement of tissue components; thus time-lapse confocal microscopy is well suited to examine these events. The general approach we have taken is to label the cell surface of microglia with fluorescent probes and, subsequently, to follow the dynamic movements of these cells, as well as their interactions with other labeled cells, within live tissue slices continuously over periods of time up to 28 h (Dailey and Waite, 1999; Stence *et al.*, 2001; Grossmann *et al.*, 2002; Petersen and Dailey, 2004).

Preparation of Central Nervous System Tissue Slices

A useful method of preparing and maintaining live brain tissue slices for microscopy is based on the organotypic (roller-tube) culture technique of Gähwiler (1984) or the static filter culture technique of Stoppini and colleagues (1991). Briefly, these techniques involve rapidly removing the tissue of interest (in this case, neonatal rat or mouse hippocampus), then slicing the tissue with a manual tissue chopper (Stoelting, Chicago, IL) at a thickness of 300 to 400 μm. Others have used a vibratome or custom-built instruments akin to an egg slicer. In the case of the roller tube technique, the tissue slices are secured to an alcohol-cleaned coverslip (11 × 22 mm) with a mixture of chicken plasma (10 μL; Cocalico) and bovine thrombin (10 μL; Sigma). Collagen gels (Vitrogen; CellTrix; O'Rourke *et al.*, 1992) and Cell-Tak (BioPolymers Inc.; Barber *et al.*, 1993) have also been used successfully to attach slices. In the case of the plasma clot, the slices are adherent within about 10 min, at which point the coverslips are placed in a test tube with 1 mL of HEPES-buffered culture media containing 25% serum. The tubes are kept in a warm box (37°C) and rotated at 12 rph in a roller drum tilted at 5° to the horizontal. In the case of the static filter cultures, brain slices are placed on porous cell culture inserts in 6-well plates containing ~1 mL of bicarbonate-buffered culture media per well. The filter cultures are maintained at 36°C in a 5% CO_2 incubator. In either case, these "organotypic" culture methods provide a means for maintaining tissue slices *in vitro* for up to several weeks (Gähwiler *et al.*, 1997).

Fluorescent Staining

Microglia

Often it is most useful to label only a small percentage of the total number of cells within a tissue volume, and in certain cases it is desirable to label only an identified subset of cells. Vital fluorescent probes must be non-toxic and resistant to photobleaching. In the case of microglia, there are several commercially available fluorescent conjugates (FITC, Alexa Fluor–488, –568, or –647) of a highly selective, non-toxic lectin (IB_4) derived from *Griffonia simplicifolia* seeds (Sigma; Molecular Probes). This lectin (IB_4) has an exclusive affinity for α-D-galactosyl sugar residues on glycoproteins and glycolipids (Wood *et al.*, 1979). In mammalian brain tissues, IB_4 labels only microglia and endothelial cells lining blood vessels and capillaries (Streit and Kreutzberg, 1987). The simultaneous labeling of microglial cell populations and blood vessels has revealed novel, dynamic interactions between these structures (Grossmann *et al.*, 2002). Incubation of brain slices or slice cultures for 1 h in IB_4-containing medium (5 μg/mL) is sufficient for robust labeling of microglia up to ~50 μm deep within tissues (Kurpius and Dailey, 2005).

Nuclei of Live or Dead Cells

One of a variety of fluorescent DNA-binding dyes is used to label live or dead cell nuclei in brain tissue slices (Dailey and Waite, 1999; Petersen and Dailey, 2004). To visualize the nuclei of live cells, we use one of the membrane-permeant dyes that have spectra in the far-red, SYTO59 (abs/em: 622/645 nm) or SYTO61 (620/647 nm). For labeling the nuclei of dead cells, we use one of the membrane impermeant dyes, Sytox Green (504/523 nm), Sytox Orange (547/570 nm), or To-Pro-3 (642/661 nm) (all from Molecular Probes). All DNA-binding dyes are applied for 10 to 20 min (1 : 10,000). These dyes are used in combination with fluorescently tagged IB_4 to image microglial behaviors in relation to other cells.

Maintaining Tissue Health on the Microscope Stage

Image data obtained from compromised tissue is useless at best, and deceiving at worst. For example, CNS slice physiologists have long known that oxygen deprivation can have severe effects on synaptic activity, although CNS tissues from developing animals seem to have a fairly high resistance to hypoxia (Dunwiddie, 1981).

It is not always easy to assess the health of living tissue on the microscope stage, but in the case of dynamic processes such as cell division or cell migration, one would at least expect that the cells perform these activities at rates near that expected based on other methods of determination. Also, one should become suspicious if the rate of activity consistently increases or decreases over the imaging session. For example, exposure of fluorescently labeled axons to high light levels can reduce the rate of extension or cause retraction. In contrast, high light levels can produce a long-lasting increase in the frequency of Ca^{2+} spikes in Fluo-3-labeled astrocytes in cultured brain slices. In many cases, there will not be a useful benchmark for determining phototoxic effects, but consis-

tent changes during imaging will serve to warn the concerned microscopist. It may be worth sacrificing a few well-labeled preps to determine if different imaging protocols, such as lower light levels or longer time intervals between images, significantly alter the biological activity under study.

Requirements for maintaining healthy tissue during imaging dictate specimen chamber design. A closed specimen chamber has the advantage of preventing evaporation during long experiments and stabilizing temperature fluctuations caused by this evaporation. We found that microglia in tissue slices maintained in a closed chamber (volume ~1 mL) with HEPES-buffered culture medium remain viable and vigorous for about 6 h, after which point the chamber medium acidifies and cell motility declines. However, when the old chamber medium is exchanged with fresh medium, the cells jump back to life again. This crude method of periodic medium exchange has supported continuous observation of DiI-labeled migrating cells in tissue slices on the microscope stage for as long as 45 h (O'Rourke *et al.*, 1992). However, when using this approach, one runs the risk of mechanically disturbing the chamber or inducing a temperature change and thereby causing a jump in focus.

A more elegant method for medium exchange and introduction of reagents involves continuous superfusion. A variety of perfusion chambers with either open or closed configurations are available (see Table 19.3). Sometimes it is necessary to design and construct very sophisticated temperature and fluid-level control systems (e.g., Delbridge *et al.*, 1990; or Walcerz and Diller, 1991). Such chambers permit very rapid exchange of medium (seconds rather than minutes), which is necessary for physiological experiments requiring high time resolution. There are also now commercially available, programmable, automated perfusion systems that permit rapid switching between one of several perfusion channels (see Table 19.3). Some experimental conditions require only relatively simple, low-cost chambers and perfusion systems, such as the one depicted in Figure 19.3 (see also Dailey *et al.*, 2005). We have used such a design to continuously superfuse tissue slices on the microscope stage for many hours. The tissues seem to remain healthy for at least 20 h when perfused (10–20 mL/h) with either the culture medium (see Dailey *et al.*, 1994) or normal saline, both of which are buffered with 25 m*M* HEPES.

Specimen heating is essential for many experiments, but this can induce an agonizing battle with focus stability (see below) as the chamber and stage components heat up. Because there is always a time lag between when the sensor of the temperature control detects that the temperature is too high (or low) and the time that the heater is able to warm the whole stage, the actual temperature of most stage heaters is always slowly oscillating, a fact that causes the focus plane to shift in a periodic manner. A sufficient period of preheating can sometimes reduce this problem. Another approach is to use a modified hair dryer to blow warm air onto both the chamber and the stage (see Dailey *et al.*, 2005) or an egg-incubator heater to heat all the air in an insulated box surrounding the entire microscope (Potter, 2004).

It is also important to monitor the temperature of the perfusing medium very near to the specimen. A low-cost microprocessor temperature controller that reduces fluctuations in the heating/cooling cycle can be obtained from Omega Engineering (Stamford, CT).

Imaging Methods

It should by now be evident that a primary concern when imaging living cells is photon collection efficiency. This is especially true when imaging dynamic processes, such as cell migration, over long periods of time. Higher collection efficiency will afford effectively lower excitation light levels, thus permitting more frequent sampling or observations of longer duration.

We have been using commercially available laser scanning confocal systems (Leica TCS NT and Leica SP2 AOBS). For illumination, the microscopes are equipped with multiple lasers. Both systems offer simultaneous excitation and detection in more than one epi-fluorescent channel. In addition, the SP2 acousto-optic

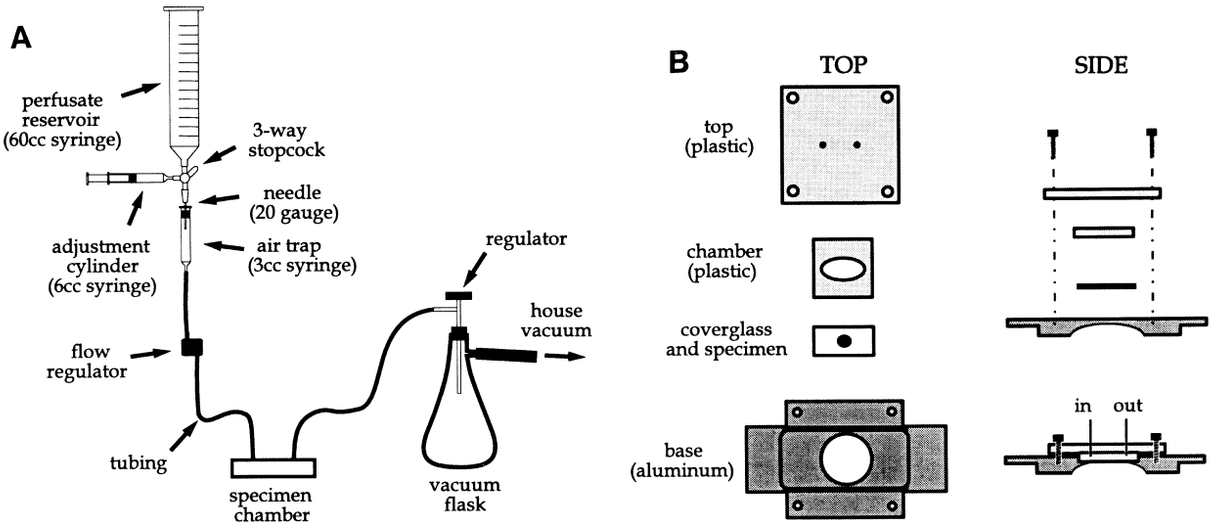

FIGURE 19.3. Schematic diagram of a simple perfusion system for maintaining tissue slices on the microscope stage. The gravity perfusion system is constructed using readily available syringes, three-way stopcocks, and tubing. The perfusion rate is adjusted using a simple thumb-wheel regulator. Perfusate is removed through a vacuum line and flask.

beam splitter (AOBS) system offers increased flexibility by providing continuously variable wavelength selection in the excitation pathway. This permits customizing the spectral detection to maximize throughput in separate channels. The Leica objectives that we find most useful are a dry 20×/0.75 PlanApo lens, and a 63×/1.2 water-immersion PlanApo objective lens.

Imaging Deep Within Tissue

Often the goal of studies in tissue slices is to examine biological processes within a cellular environment that approximates that found *in situ*. In the case of tissue slices, it is usually desirable to image as far from cut tissue surfaces as possible to avoid artifacts associated with tissue damage. However, the cut surfaces of developing CNS tissue slice cultures contain a plethora of astrocytes, activated microglia, and a mat of growing neuronal processes. Time-lapse imaging of these regions provides striking footage of glial cell movements, proliferation, and phagocytosis (Stence *et al.*, 2001; Petersen and Dailey, 2004).

If high-NA (1.3, 1.4) oil-immersion lenses are used to focus more than 5–10 μm into most living specimens, spherical aberration will severely reduce resolution and consequently diminish signal levels (see also Chapters 1, 7 and 20, *this volume*). Instead one should use water-immersion lenses, or on tissue having a refractive index (RI) around 1.48, an objective designed for glycerin immersion. Table 18.2 in the preceding Chapter lists RI values for a number of tissues. Efforts to image more than 50μm into tissue is also complicated by a number of other factors:

- Weak staining of cells due to poor dye penetration.
- Light scatter by the overlying tissue components.
- Spherical aberration.

The first problem can be overcome if the dye can be injected into the tissue with a minimum of disruption, or if tissues can be harvested from transgenic animals expressing fluorescent proteins in subsets of cells (e.g., Zuo *et al.*, 2004). Light scatter by the tissue can be minimized by using longer-wavelength dyes (see Chapters 28 and 37, *this volume*). Indeed, we find that a long-wavelength (Alexa Fluor-647; Molecular Probes) conjugate of IB_4 noticeably improves visibility of labeled cells in deeper portions of the tissue slice. Imaging at longer wavelengths may also reduce phototoxic effects because the light is of lower energy. Finally, the problem of spherical aberration can be improved by using water-immersion objective lenses or the recently introduced automatic spherical aberration corrector (Intelligent Imaging Innovations, Denver, CO; and also Chapters 1, 7, and 20, *this volume*).

Keeping Cells in Focus

The optical sectioning capability of the confocal microscope can be simultaneously a blessing and a curse. On the one hand, thin optical sections reduce out-of-focus flare and improve resolution. However, with such a shallow depth of focus, even very small changes in the position of the objective lens relative to the object of interest within the specimen can ruin an otherwise perfect experiment. This is a particular problem when imaging thin, tortuous structures such as axons or dendritic spines within neural tissue. A moving focal plane can, for example, give one the erroneous impression of dendritic spine extension or retraction. This problem is compounded when imaging cells and cell processes that are in fact actively moving within the tissue.

One obvious approach is to image the cells in four dimensions (3D × time). This can absorb some changes in tissue and stage movement as well as help track cells that move from one focal plane to another. In addition, our strategy has been to image with the detector pinhole aperture substantially open, corresponding to a pinhole size roughly 4 Airy units in size. Although this reduces the axial resolution slightly, it has the dual advantage of achieving a higher S/N at a given illumination intensity, as well as thickening the optical section. On our microscope systems, the open pinhole configuration gives an apparent optical section thickness of about 3 μm when using a 20× NA 0.7 objective. Thus, for each time point, we collect about 15 images at axial step intervals of ~2 μm. The guiding principle here is to space the image planes in the axial dimension so as to maximize the volume of tissue imaged but not lose continuity between individual optical-section images.

When these image stacks are collected at ~5-min intervals at power levels of ~50 to 75 μW (back aperture of objective), IB_4-labeled cells do not appear to suffer phototoxic effects and can be imaged continuously for over 20 h. Image stacks can be recombined later using a maximum brightness operation. Unfortunately, even when *z*-axis stacks of images are collected, tissue movements can be so severe as to necessitate a continuous "tweaking" of the focus. Thus, it is helpful to monitor image features on the screen (by making fiduciary marks on an acetate sheet taped to the monitor screen), or to store the data in such a way that they are quickly accessible and can be reviewed on the fly to make corrective focus adjustments.

Ideally, one would like an automated means of maintaining the desired plane of focus, especially for long imaging sessions. Although there are several autofocus methods that work for simple specimens (e.g., Firestone *et al.*, 1991), imaging structures in 3D tissue presents a significant challenge because there is no single image plane on which to calculate focus.

Handling the Data

Imaging tissue in 3D over time solves some problems but generates others. In particular, how does one deal with the gigabytes of image data that are often obtained in a single experiment? Fortunately, improvements in desktop computer performance and storage capacity, coupled with lower cost, make this much less of a problem than it used to be. Desktop workstations now contain hard drives with very large storage capacities (hundreds of gigabytes), and archiving methods and media are readily accessible and inexpensive. We typically store newly acquired data on a network server for image processing (such as spatial filtering or deconvolution) and analysis, then archive the data onto compact disks (CDs) or digital video disks (DVDs). This is a technology that is sure to continue rapid advancement, making it easier to store and access image data. At the end of the time-lapse experiment, the *z*-axis image stacks are combined in a variety of ways for viewing. For time-lapse studies, it is generally most useful to produce a set of "extended focus" images for viewing time-points in rapid succession. Depending on the file format, these image series can be viewed in one of a number of image viewers, including Scion Image or ImageJ (freely available from Scion Corp.). To create the projection images, we use a maximum brightness operation running in a custom-written Pascal macro to construct a 2D representation of each 3D data set (Stence *et al.*, 2001). When the axial step interval is appropriate (see above), portions of single cells that pass through the various focal planes appear contiguous. Alternatively, the image stacks can be reassembled into a set of

red–green 3D stereo images. Such images, when played in rapid succession, provide 3D depth information as well as time information in thick tissue samples. If more than one fluorescent channel is used, they can be combined to create multicolor images.

Results

The ability to collect 3D image data sets over long periods of time and at relatively short time intervals has revealed new information on the dynamics of microglial cell activation, migration, and cell–cell interactions in live mammalian brain tissues. Here we offer some examples of the kinds of data that can be obtained, along with some possible modes of data analysis.

In Figure 19.4, we show an example of a one-channel, single focal plane confocal time-lapse observation. Several IB_4-labeled microglial cells in a live, "roller tube" slice culture were repeatedly imaged at intervals of a few minutes to reveal movements of microglial cells over time. These movements can be quantified using automated edge detection [Fig. 19.4(A)]. Computer analysis using 2D Dynamic Image Analysis Software (DIAS; Soll, 1995) shows differences in motile behavior of three cells, as determined by tracing the cell perimeter [Fig. 19.4(B)]. Cell motility movements can be displayed as regions of protrusion (green) or retraction (red) by comparing cell shape over successive time-points [Fig. 19.4(C)]. Many parameters of cell shape and movement can be quantified easily using DIAS (Soll, 1995, 1999; Heid *et al.*, 2002).

Dual-channel, time-lapse confocal imaging of a specimen labeled with more than one fluorophore can provide information on the dynamic relationship between cell components or diverse cell types. In Figure 19.5, we show a two-channel time-lapse series of IB_4-labeled microglia and cell nuclei. Images from each channel (green and red) were collected simultaneously using a two-detector system. The nuclear marker (SYTO 61) labels all cells, including microglia. Time-lapse imaging shows the location of nuclei within migrating cells [Fig. 19.5(A)], and DIAS analysis can be used to plot and analyze the movements of nuclei [Fig. 19.5(B)].

Dual-channel, 3D time-lapse confocal imaging can yield information on the relative movement and location of structures labeled with two or more fluorophores within a thick tissue specimen. In the example shown in Figure 19.6, the movements of IB_4-labeled microglia are observed in the context of dead cell nuclei labeled with To-Pro-3, a membrane-impermeant DNA binding dye. The stereo images reveal the movements of microglia on the surface of the tissue slice as well as deep within the tissue. Such information is being used to study differences in the behaviors and cell–cell interactions of microglia in a brain tissue environment. Note, for example, the rapid microglial movement and phagocytosis of a dead cell nucleus in the time-lapse series [Fig. 19.6(D)].

In summary, the 4D and five-dimensional (5D) imaging methods outlined here capture more of the events occurring within the tissue and also provide the researcher with assurance that apparent changes in the length of even the smallest branches and fine filopodia are not due to the structures moving in and out of the focal plane. They also provide a means for exploring the dynamic interactions between different molecules, cells, and cell types within thick biological specimen.

Conclusion

Imaging of living specimens at a high S/N can be achieved with very low levels of incident illumination.

Imaging with a fairly large pinhole strikes a balance between image quality and focus problems during long-term observation of live tissue.

Four-dimensional time-lapse imaging is useful for following moving cells and fine cellular processes within complex tissues. Under optimal conditions, 3D data can be collected over long periods of time (>20 h) at relatively short time intervals (~5 min).

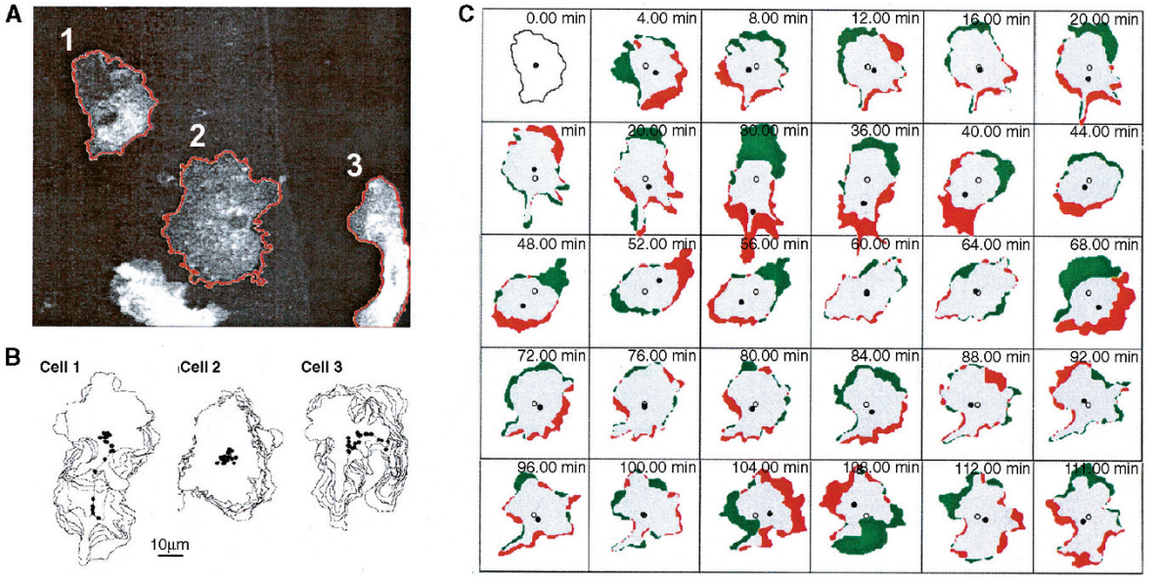

FIGURE 19.4. Use of a Dynamic Image Analysis System (DIAS; Soll, 1995, 1999) to characterize microglial motility behaviors in time-lapse imaging experiments. (A) Boundaries (*red lines*) of three FITC–IB_4-labeled microglial cells were defined by automated, computer-assisted edge detection. (B) Tracings of the three cells show motility behaviors over a 2 h period. The cell centroid (*black dots*) were computed and plotted for each time-point. Note that all three cells are motile, but cell 2 does not locomote. (C) Areas of new protrusion (*green*) and resorption (*red*) are shown for cell 1 at 4 min intervals. The new centroid (*open dot*) is shown in relation to the former centroid (*filled dot*).

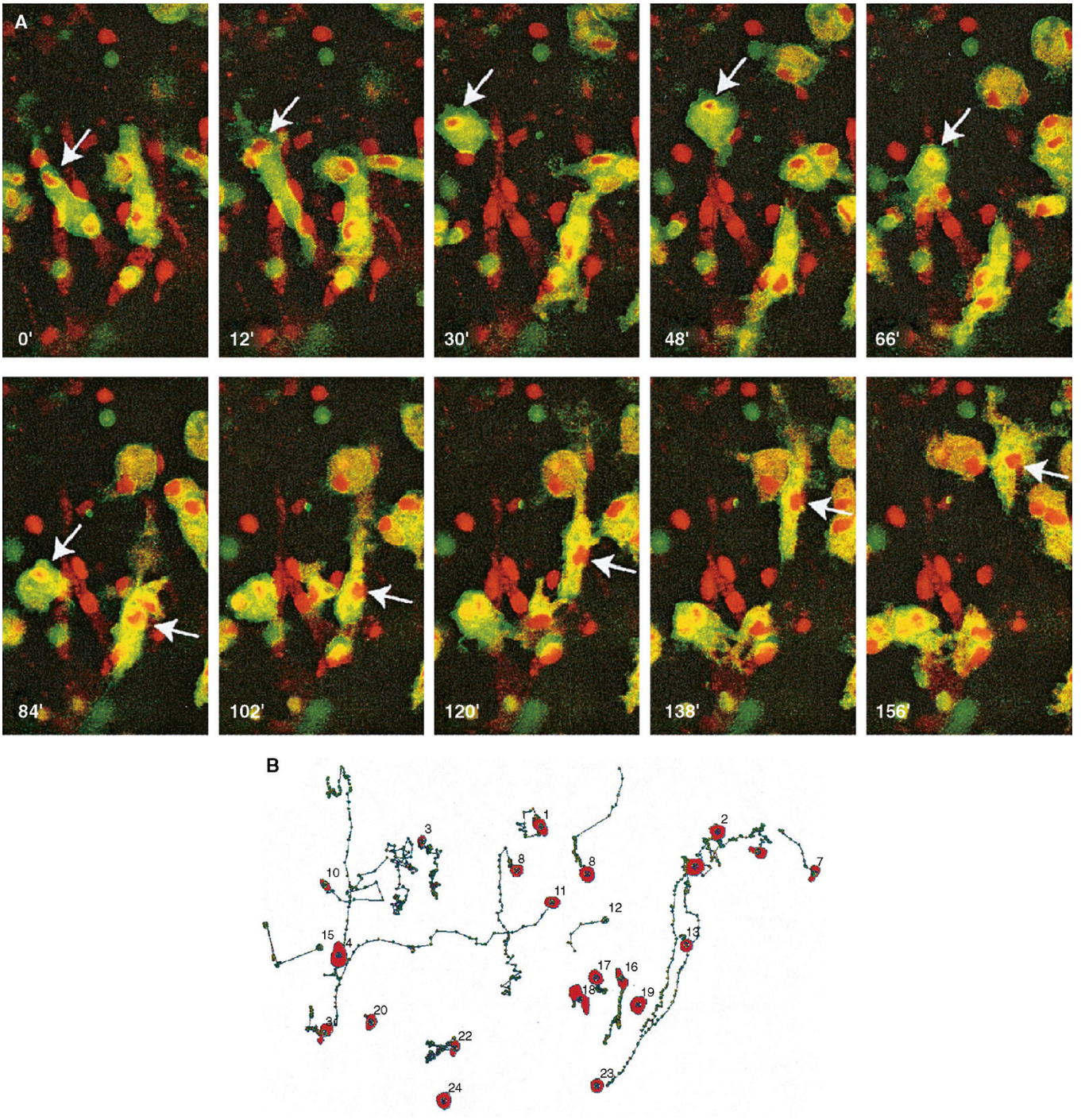

FIGURE 19.5. Two-channel time-lapse imaging of microglia (*green*) and cell nuclei (*red*). (A) Time lapse sequence shows capability of simultaneously imaging IB_4-labeled microglia and nuclei of cells using a cell membrane permeant DNA binding dye (Syto61). Note the nuclei (*arrows*) in two different migrating microglial cells. The sequence spans 156 min. Only a small portion of the original field of view is shown. (B) Automated tracing of the movement of cell nuclei shows paths taken by nuclei of cells in the experiment show in (A). Nuclei were detected by DIAS software (Soll, 1995). Only a select subset of nuclei are shown. Note differences in the movement among different cells.

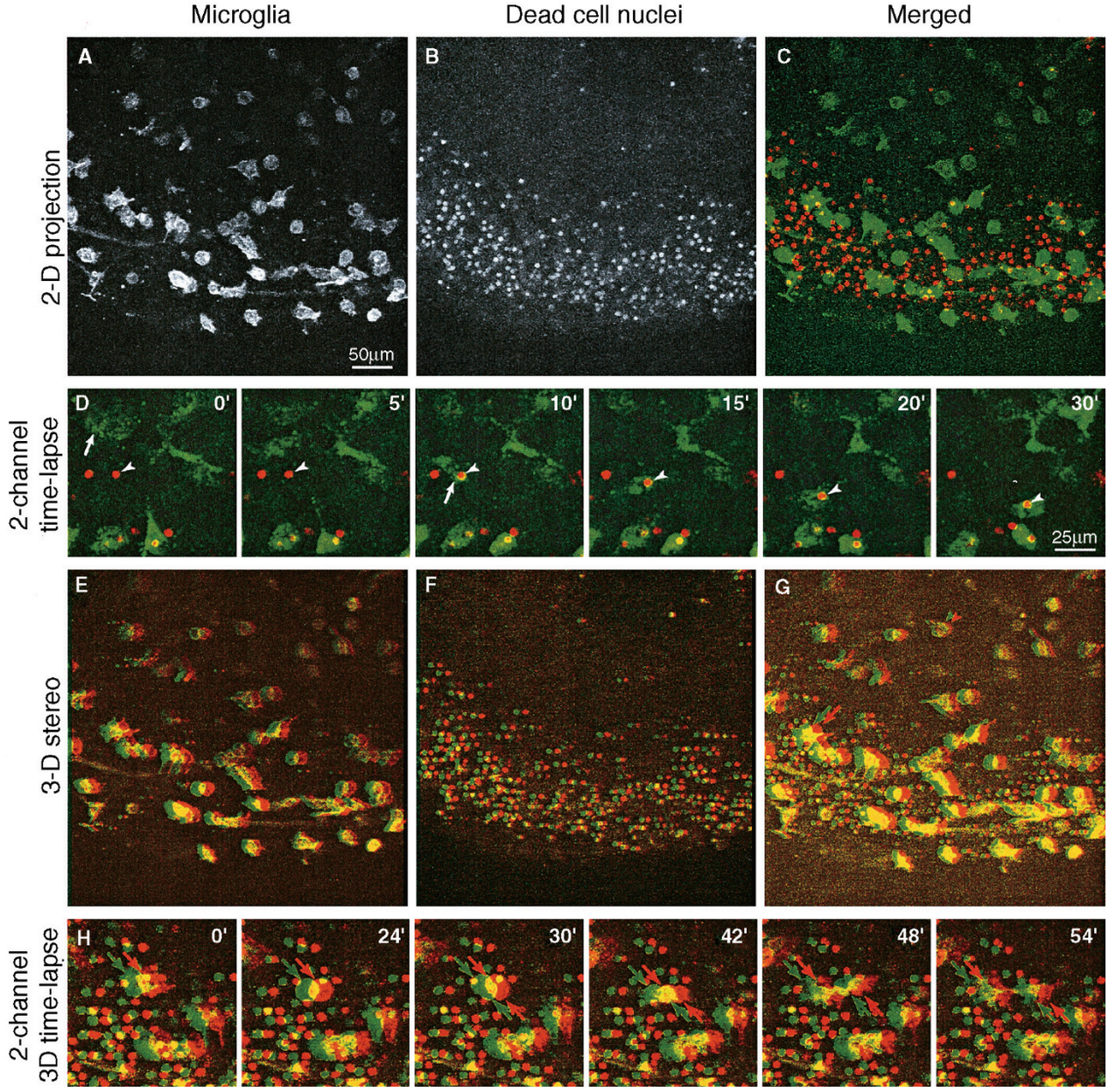

FIGURE 19.6. Two- and three-dimensional, two-channel time-lapse imaging of microglial movements and phagocytosis of dead cell nuclei in live rat brain tissue slice cultures (P6+2DIV). (A) Microglia are stained with a fluorescent lectin, FITC-IB_4. (B) Dead cell nuclei are stained with To-Pro-3. (C) Merged image of (A) and (B). The image represents a projection of nine optical sections spanning 40 μm in *z*-depth. Images were captured using a 20×/0.7 dry objective. (D) Time-lapse sequence of a portion of the field of view above showing phagocytic clearance of a dead cell nucleus (*arrowhead*) by a locomotory microglial cell (*arrow*). Note that the microglial cell maintains a rapid rate of locomotion as it sweeps over and picks up the dead cell nucleus. (E–G) 3D stereo projections of images in A through C above. Use red–green glasses (red over left eye) to view depth. (H) 3D stereo time-lapse sequence showing mitosis of a microglial cell (*arrow*) near the surface of the brain slice culture. The small, round objects represent the condensed nuclei of dead cells. Time is shown in minutes. See the supplemental video movies at http://www.springer.com/0-387-25921-X (Adapted from Petersen and Dailey, 2004.)

Multi-channel (5D) imaging can provide information on the dynamic relationship of different cell or tissue components. Future developments should address constraints on high-resolution imaging deep (>50 μm) within tissue. Improvements will likely be achieved by using water-immersion lenses and external automatic spherical aberration correctors (see Chapter 20, *this volume*) and by employing longer wavelength dyes to reduce light scatter by the tissue and to minimize phototoxic effects.

FUTURE DIRECTIONS

For confocal microscopy of living cells, the most important characteristic of the instrument is its efficiency in collecting and detecting the fluorescence emission light from the specimen (Chapter 2, *this volume*). Any improvement in this efficiency reduces the amount of light damage and allows the gathering of more data. The increased data can either be in the form of more images, images

with less statistical noise, or images obtained with greater spatial or temporal resolution. Newer models of existing commercial confocal microscopes have substantially improved photon efficiency. In addition, there have been technological improvements in the ability to separate the excitation and fluorescence emission of fluorophores, providing greater flexibility for multi-channel imaging and quantitative image analysis in live cells and tissues. Finally, the advantages of either Gaussian-filtering 2D data or deconvolving 3D data to reduce the effects of Poisson noise are now widely appreciated. Routine application of this approach can reduce the light load to the specimen by a factor of from 10 to 100 while still producing images with the same apparent resolution and signal-to-noise ratio.

Technological and conceptual advancements are also likely to push the spatial and temporal resolution and other modes of fluorescence microscopy (e.g., Gustafsson, 1999; Hell *et al.*, 2004; Chapters 13, 30, and 31, *this volume*). Some of these approaches (e.g., 4Pi-microscopy) look promising for live cells (Gugel *et al.*, 2004), but their potential for widespread use in biological applications has yet to be established, and there are limitations on the sample thickness (Gustafsson, 1999 and Chapter 21, *this volume*). In addition, higher resolution implies smaller pixels and therefore more photons/square micrometer and more bleaching and toxicity. Undoubtedly, there will be more improvements and wider applications along these lines in the future.

Although it is difficult to predict the future of confocal microscopy of living cells, as confocal microscopy (and its richer cousin, multi-photon microscopy) are in all probability the optimal methods for studying the 3D structure of living cells, the future seems sure to be bright!

ACKNOWLEDGMENTS

The authors thank Glen MacDonald for helpful information on autofluorescence and light sensitivity of culture media, and for bringing relevant literature to our attention. MED thanks the staff of the W.M. Keck Motion Analysis Facility in the Department of Biological Sciences, University of Iowa, for their assistance in obtaining the data shown in Figures 19.4 and 19.5. Data for Figures 19.4 and 19.5 were kindly provided by Marc Waite. Data for Figure 19.6 were kindly provided by Mark Petersen.

REFERENCES

Adams, C.L., Chen, Y.T., Smith, S.J., and Nelson, W.J., 1998, Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein, *J. Cell Biol.* 142:1105–1119.

Adams, M.C., Salmon, W.C., Gupton, S.L., Cohan, C.S., Wittmann, T., Prigozhina, N., and Waterman-Storer, C.M., 2003, A high-speed multispectral spinning-disk confocal microscope system for fluorescent speckle microscopy of living cells, *Methods* 29:29–41.

Amos, W.B., Reichelt, S., Cattermole, D.M., and Laufer, J., 2003, Re-evaluation of differential phase contrast (DPC) in a scanning laser microscope using a split detector as an alternative to differential interference contrast (DIC) optics, *J. Microsc.* 210:166–175.

Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A., 2002, An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein, *Proc. Natl. Acad. Sci. USA* 99:12651–12656.

Ando, R., Mizuno, H., and Miyawaki, A., 2004, Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting, *Science* 306:1370–1373.

Andrews, P.M., Petroll, W.M., Cavanagh, H.D., and Jester, J.V., 1991, Tandem scanning confocal microscopy (TSCM) of normal and ischemic living kidneys, *Am. J. Anat.* 191:95–102.

Andrews, P.D., Harper, I.S., and Swedlow, J.R., 2002, To 5D and beyond: Quantitative fluorescence microscopy in the postgenomic era, *Traffic* 3:29–36.

Ashkin, A., Schutze, K., Dziedzic, J.M., Euteneuer, U., and Schliwa, M., 1990, Force generation of organelle transport measured *in vivo* by an infrared laser trap, *Nature* 348:346–348.

Ashworth, R., 2004, Approaches to measuring calcium in zebrafish: Focus on neuronal development, *Cell Calcium* 35:393–402.

Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E., and Webb, W.W., 1976, Mobility measurement by analysis of fluorescence photobleaching recovery kinetics, *Biophys. J.* 16:1055–1069.

Bacia, K., and Schwille, P., 2003, A dynamic view of cellular processes by in vivo fluorescence auto- and cross-correlation spectroscopy, *Methods* 29:74–85.

Baker, G.E., and Reese, B.E., 1993, Using confocal laser scanning microscopy to investigate the organization and development of neuronal projections labeled with DiI, *Methods Cell Biol.* 38:325–344.

Baker, M.W., Kauffman B., Macagno, E.R., and Zipser, B., 2003, *In vivo* dynamics of CNS sensory arbor formation: a time-lapse study in the embryonic leech, *J. Neurobiol.* 56:41–53.

Ballestrem, C., Hinz, B., Imhof, B.A., Wehrle-Haller, B., 2001, Marching at the front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior, *J. Cell Biol.* 155(7):1319–1332.

Barber, R.P., Phelps, P.E., and Vaughn, J.E., 1993, Preganglionic autonomic motor neurons display normal translocation patterns in slice cultures of embryonic rat spinal cord, *J. Neurosci.* 13:4898–4907.

Bastiaens, P.I., and Pepperkok, R., 2000, Observing proteins in their natural habitat: The living cell, *Trends Biochem. Sci.* 25:631–637.

Bastiaens, P.I., and Squire, A. 1999, Fluorescence lifetime imaging microscopy: Spatial resolution of biochemical processes in the cell, *Trends Cell Biol.* 9:48–52.

Baumgartner, W., Schutz, G.J., Wiegand, J., Golenhofen, N., and Drenckhahn, D., 2003, Cadherin function probed by laser tweezer and single molecule fluorescence in vascular endothelial cells, *J. Cell Sci.* 116:1001–1011.

Bement, W.M., Sokac, A.M., and Mandato, C.A., 2003, Four-dimensional imaging of cytoskeletal dynamics in *Xenopus* oocytes and eggs, *Differentiation* 71:518–527.

Benediktsson, A.M., Schachtele, S.J., Green, S.H., and Dailey, M.E., 2005, Ballistic labeling and dynamic imaging of astrocytes in organotypic hippocampal slice cultures, *J. Neurosci. Methods* 141:41–53.

Berg, R.H., 2004, Evaluation of spectral imaging for plant cell analysis, *J. Microsc.* 214(Pt 2):174–181.

Betz, W.J., Mao, F., and Bewick, G.S., 1992, Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals, *J. Neurosci.* 12:363–375.

Block, S.M., Goldstein, L.S., and Schnapp, B.J., 1990, Bead movement by single kinesin molecules studied with optical tweezers, *Nature* 348:348–352.

Bloom, J.A., and Webb, W.W., 1984, Photodamage to intact erythrocyte membranes at high laser intensities: methods of assay and suppression, *J. Histochem. Cytochem.* 32:608–616.

Brum, G., Gonzalez, A., Rengifo, J., Shirokova, N., and Rios, E., 2000, Fast imaging in two dimensions resolves extensive sources of Ca^{2+} sparks in frog skeletal muscle, *J. Physiol.* 528:419–433.

Brustein, E., Marandi, N., Kovalchuk, Y., Drapeau, P., and Konnerth, A., 2003, "In vivo" monitoring of neuronal network activity in zebrafish by two-photon Ca(2+) imaging, *Pflugers Arch.* 446(6):766–773.

Cahalan, M.D., Parker, I., Wei, S.H., and Miller, M.J., 2002, Two-photon tissue imaging: Seeing the immune system in a fresh light, *Nat. Rev. Immunol.* 2:872–880.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C., 1994, Green fluorescent protein as a marker for gene expression, *Science* 263:802–805.

Chen, Y., Mills, J.D., and Periasamy, A., 2003, Protein localization in living cells and tissues using FRET and FLIM, *Differentiation* 71:528–541.

Cechin, S.R., Gottfried, C., Prestes, C.C., Andrighetti, L., Wofchuk, S.T., and Rodnight, R., 2002, Astrocyte stellation in saline media lacking bicarbon-

ate: possible relation to intracellular pH and tyrosine phosphorylation, *Brain Res*. 946:12–23.

Cleemann, L., Wang, W., and Morad, M., 1998, Two-dimensional confocal images of organization, density and gating of focal Ca^{2+} release sites in rat cardiac myocytes, *Proc. Natl. Acad. Sci. USA* 95:10984–10989.

Cogswell, C.J., and Sheppard, C.J.R., 1991, Visualization of 3-D phase structure in confocal and conventional microscopy, *Proc. SPIE*. 1450:323–328.

Cogswell, C.J., and Sheppard, C.J.R., 1992, Confocal differential interference contrast (DIC) microscopy: Including a theoretical analysis of conventional and confocal DIC imaging, *J. Microsc*. 165:81–101.

Cooper, M.S., Cornell-Bell, A.H., Chernjavsky, A., Dani, J.W., and Smith, S.J., 1990, Tubulovesicular processes emerge from trans-Golgi cisternae, extend along microtubules, and interlink adjacent trans-Golgi elements into a reticulum, *Cell* 61:135–145.

Cooper, M.S., D'Amico, L.A., and Henry, C.A., 1999, Confocal microscopic analysis of morphogenetic movements, *Methods Cell Biol*. 59:179–204.

Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S., and Smith, S.J., 1990, Glutamate induces calcium waves in cultured astrocytes: Long-range glial signaling, *Science* 247:470–473.

Crittenden, S.L., and Kimble J., 1999, Confocal methods for *Caenorhabditis elegans*, *Methods Mol Biol*. 122:141–151.

Dailey, M.E., and Smith, S.J., 1993, Confocal imaging of mossy fiber growth in live hippocampal slices, *Jpn. J. Physiol*. 43:S183–S192.

Dailey, M.E., and Smith, S.J., 1994, Spontaneous Ca^{2+} transients in developing hippocampal pyramidal cells, *J. Neurobiol*. 25:243–251.

Dailey, M.E., and Smith, S.J., 1996, The dynamics of dendritic structure in developing hippocampal slices, *J. Neurosci*. 16:2983–2994.

Dailey, M.E., Buchanan, J., Bergles, D.E., and Smith, S.J., 1994, Mossy fiber growth and synaptogenesis in rat hippocampal slices *in vitro*, *J. Neurosci*. 14:1060–1078.

Dailey, M.E., and Waite, M., 1999, Confocal imaging of microglial cell dynamics in hippocampal slice cultures, *Methods* 18:222–230.

Dailey, M.E., Marrs, G.S., and Kurpius, D., 2005, Maintaining live cells and tissue slices in the imaging setup, In: *Imaging in Neuroscience and Development* (R. Yuste and A. Konnerth, eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Das, T., Payer, B., Cayouette, M., and Harris, W.A., 2003, *In vivo* time-lapse imaging of cell divisions during neurogenesis in the developing zebrafish retina, *Neuron* 37:597–609.

Delbridge, L.M., Harris, P.J., Pringle, J.T., Dally, L.J., and Morgan, T.O., 1990, A superfusion bath for single-cell recording with high-precision optical depth control, temperature regulation, and rapid solution switching, *Pfluegers Arch*. 416:94–97.

Del Pozo, M.A., Kiosses, W.B., Alderson, N.B., Meller, N., Hahn, K.M., Schwartz, M.A., 2002, Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI, *Nat. Cell Biol.* 4(3):232–239.

Dickinson, M.E., Bearman, G., Tille, S., Lansford, R., Fraser, S.E., 2001, Multispectral imaging and linear unmixing add a whole new dimension to laser scanning fluorescence microscopy, *Biotechniques* 31(6):1272, 1274–1276, 1278.

Dirnagl, U., Villringer, A., and Einhaupi, K.M., 1992, *In vivo* confocal scanning laser microscopy of the cerebral microcirculation, *J. Microsc*. 165:147–157.

Dixit, R., and Cyr, R., 2003, Cell damage and reactive oxygen species production induced by fluorescence microscopy: Effect on mitosis and guidelines for non-invasive fluorescence microscopy, *Plant J*. 36:280–290.

Dorman, G., and Prestwich, G.D., 2000, Using photolabile ligands in drug discovery and development, *Trends Biotechnol*. 18:64–77.

Dunn, G.A., Dobbie, I.M., Monypenny, J., Holt, M.R., and Zicha, D., 2002, Fluorescence localization after photobleaching (FLAP): A new method for studying protein dynamics in living cells, *J. Microsc*. 205:109–112.

Dunwiddie, T.V., 1981, Age-related differences in the *in vitro* rat hippocampus: Development of inhibition and the effects of hypoxia, *Dev. Neurosci*. 4:165–175.

Dvorak, J.A., and Stotler, W.F., 1971, A controlled-environment culture system for high resolution light microscopy, *Exp. Cell Res*. 68:269–275.

Dynes, J.L., and Ngai, J., 1998, Pathfinding of olfactory neuron axons to stereotyped glomerular targets revealed by dynamic imaging in living zebrafish embryos, *Neuron* 20:1081–1091.

Edwards, A.M., Silva, E., Jofre, B., Becker, M.I., and De Ioannes, A.E., 1994, Visible light effects on tumoral cells in a culture medium enriched with tryptophan and riboflavin, *J. Photochem. Photobiol*. B 24:179–186.

Elangovan, M., Day, R.N., and Periasamy, A., 2002, Nanosecond fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy to localize the protein interactions in a single living cell, *J. Microsc*. 205:3–14.

Ellenberg, J., Lippincott-Schwartz, J., and Presley, J.F., 1999, Dual-colour imaging with GFP variants, *Trends Cell Biol*. 9:52–56.

Elson, E.L., 2001, Fluorescence correlation spectroscopy measures molecular transport in cells, *Traffic* 2:789–796.

Fan, G.Y., Fujisaki, H., Miyawaki, A., Tsay, R.K., Tsien, R.Y., and Ellisman, M.H., 1999, Video-rate scanning two-photon excitation fluorescence microscopy and ratio imaging with chameleons, *Biophys. J*. 76:2412–2120.

Firestone, L., Cook, K., Culp, K., Talsania, N., and Preston, K. Jr., 1991, Comparison of autofocus methods for automated microscopy, *Cytometry* 12:195–206.

Flock, A., Flock, B., and Scarfone, E., 1998, Laser scanning confocal microscopy of the hearing organ: fluorochrome-dependent cellular damage is seen after overexposure, *J. Neurocytol*. 27:507–516.

Forsythe, I.W., 1991, Microincubator for regulating temperature and superfusion of tissue-cultured neurons during electrophysiological or optical studies, *Methods Neurosci*. 4:301–318.

Fraser, S.E., and O'Rourke, N.A., 1990, *In situ* analysis of neuronal dynamics and positional cues in the patterning of nerve connections, *J. Exp. Biol*. 153:61–70.

Frostig, R., ed., 2002, In Vivo *Optical Imaging of Brain Function*, CRC Press, Boca Raton, Florida.

Gahtan, E., Sankrithi, N., Campos, J.B., and O'Malley, D.M., 2002, Evidence for a widespread brain stem escape network in larval zebrafish, *J. Neurophysiol.* 87(1):608–614.

Gähwiler, B.H., 1984, Development of the hippocampus *in vitro*: Cell types, synapses, and receptors, *Neuroscience* 11:751–760.

Gähwiler B.H., Capogna M., Debanne D., McKinney R.A., and Thompson S.M., 1997, Organotypic slice cultures: A technique has come of age, *Trends Neurosci*. 20:471–477.

Gan, W.B., Kwon, E., Feng, G., Sanes, J.R., and Lichtman, J.W., 2003, Synaptic dynamism measured over minutes to months: Age-dependent decline in an autonomic ganglion, *Nat. Neurosci*. 6:956–960.

Gerlich, D., and Ellenberg, J., 2003, 4D imaging to assay complex dynamics in live specimens, *Nat. Cell Biol*. 5:S14–S19.

Gerlich, D., Beaudouin, J., Gebhard, M., Ellenberg, J., and Eils, R., 2001, Four-dimensional imaging and quantitative reconstruction to analyse complex spatiotemporal processes in live cells, *Nat. Cell Biol*. 3:852.

Gilland, E., Miller, A.L., Karplus, E., Baker. R., and Webb, S.E., 1999, Imaging of multicellular large-scale rhythmic calcium waves during zebrafish gastrulation, *Proc. Natl. Acad. Sci. USA* 96:157–161.

Gleason, M.R., Higashijima, S., Dallman, J., Liu, K., Mandel, G., and Fetcho, J.R., 2003, Translocation of CaM kinase II to synaptic sites *in vivo, Nat. Neurosci*. 6:217–218.

Goksor, M., Enger, J., and Hanstorp, D., 2004, Optical manipulation in combination with multiphoton microscopy for single-cell studies, *Appl. Opt*. 43:4831–4837.

Gong, Y., Mo, C., and Fraser, S.E., 2004, Planar cell polarity signalling controls cell division orientation during zebrafish gastrulation, *Nature* 430:689–693.

Griffin, F.M., Ashland, G., and Capizzi, R.L., 1981, Kinetics of phototoxicity of Fischer's medium for L5178Y leukemic cells, *Cancer Res*. 41:2241–2248.

Grossmann, R., Stence, N., Carr, J., Fuller, L., Waite, M., and Dailey, M.E., 2002, Juxtavascular microglia migrate along brain capillaries following activation during early postnatal development, *Glia* 37:229–240.

Gugel, H., Bewersdorf, J., Jakobs, S., Engelhardt, J., Storz, R., and Hell, S.W., 2004, Combining 4Pi excitation and detection delivers seven-fold sharper sections in confocal imaging of live cells, *Biophys. J*. 87(6):4146–4152.

Gustafsson, M., 1999, Extended resolution fluorescence microscopy, *Curr. Opin. Struct. Biol*. 9:627–634.

Hammond, A.T., and Glick, B.S., 2000, Raising the speed limits for 4D fluorescence microscopy, *Traffic* 1:935–40.

Hasan, M.T., Friedrich, R.W., Euler, T., Larkum, M.E., Giese, G., Both, M., Duebel, J., Waters, J., Bujard, H., Griesbeck, O., Tsien, R.Y., Nagai, T., Miyawaki, A., and Denk, W., 2004, Functional fluorescent Ca^{2+} indicator proteins in transgenic mice under TET control, *PLoS Biol*. 2:e163. Epub 2004 Jun 15.

Heid, P.J., Voss, E., and Soll, D.R., 2002, 3D-DIASemb: A computer-assisted system for reconstructing and motion analyzing in 4D every cell and nucleus in a developing embryo, *Dev. Biol*. 245:329–347.

Hell, S.W., Dyba, M., and Jakobs, S., 2004, Concepts for nanoscale resolution in fluorescence microscopy, *Curr. Opin. Neurobiol*. 14:599–609.

Hess, S.T., Huang, S., Heikal, A.A., and Webb, W.W., 2002, Biological and chemical applications of fluorescence correlation spectroscopy: A review, *Biochemistry* 41:697–705.

Hiraoka, Y., Shimi, T., and Haraguchi, T., 2002, Multispectral imaging fluorescence microscopy for living cells, *Cell Struct. Funct*, 27:367–374.

Hollingworth, S., Soeller, C., Baylor, S.M., and Cannell, M.B., 2000, Sarcomeric Ca^{2+} gradients during activation of frog skeletal muscle fibres imaged with confocal and two-photon microscopy, *J. Physiol*. 526:551–560.

Honig, M.G., and Hume, R.I., 1986, Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures, *J. Cell Biol*. 103:171–187.

Hook, G.R., and Odeyale, C.O., 1989, Confocal scanning fluorescence microscopy: A new method for phagocytosis research, *J. Leukoc. Biol*. 45:277–282.

Hoppe, A.D., Swanson, J.A., 2004, Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis, *Mol. Biol. Cell* 15(8):3509–3519.

Ince, C., Ypey, D.L., Diesselhoff-Den Dulk, M.M., Visser, J.A., De Vos, A., and Van Furth, R., 1983, Micro-CO_2-incubator for use on a microscope, *J. Immun. Methods* 60:269–275.

Isogai, S., Lawson, N.D., Torrealday, S., Horiguchi, M., and Weinstein, B.M., 2003, Angiogenic network formation in the developing vertebrate trunk, *Development* 130:5281–5290.

Jester, J.V., Andrews, P.M., Petroll, W.M., Lemp, M.A., and Cavanagh, H.D., 1991, *In vivo*, real-time confocal imaging, *J. Electron Microsc. Tech*. 18:50–60.

Jester, J.V., Petroll, W.M., Garana, R.M.R., Lemp, M.A., and Cavanagh, H.D., 1992, Comparison of *in vivo* and *ex vivo* cellular structure in rabbit eyes by tandem scanning microscopy, *J. Microsc*. 165:169–181.

Johnson, L.V., Walsh, M.L., and Chen, L.B., 1980, Localization of mitochondria in living cells with rhodamine 123, *Proc. Natl. Acad. Sci. USA* 77:990–994.

Jontes, J.D., Buchanan, J., and Smith, S.J., 2000, Growth cone and dendrite dynamics in zebrafish embryos: early events in synaptogenesis imaged *in vivo, Nat. Neurosci*. 3:231–237.

Kim, S.A., Heinze, K.G., Waxham, M.N., and Schwille, P., 2004, Intracellular calmodulin availability accessed with two-photon cross-correlation, *Proc. Natl. Acad. Sci. USA* 101:105–110.

Keller, P., Toomre, D., Díaz, E., White, J., and Simons, K., 2001, Multicolour imaging of post-Golgi sorting and trafficking in live cells, *Nat. Cell Biol*. 3:140–149.

Knebel, W., Quader, H., and Schnepf, E., 1990, Mobile and immobile endoplasmic reticulum in onion bulb epidermis cells: short- and long-term observations with a confocal laser scanning microscope, *Eur. J. Cell Biol*. 52:328–340.

Knight, M.M., Roberts, S.R., Lee, D.A., and Bader, D.L., 2003, Live-cell imaging using confocal microscopy induces intracellular calcium transients and cell death, *Am. J. Physiol. Cell Physiol*. 284:C1083–C10839.

Koppel, D.E., Axelrod, D., Schlessinger, J., Elson, E.L., and Webb, W.W., 1976, Dynamics of fluorescence marker concentration as a probe of mobility, *Biophys. J*. 16:1315–1329.

Koster, R.W., and Fraser, S.E., 2001, Direct imaging of *in vivo* neuronal migration in the developing cerebellum, *Curr. Biol*. 11:1858–1863.

Kuo, S.C., 2001, Review: Using optics to measure biological forces and mechanics, *Traffic* 2:757–763.

Kuo, S.C., and Sheetz, M.P., 1993, Force of single kinesin molecules measured with optical tweezers, *Science* 260:232–234.

Kurpius, D., and Dailey, M.E., 2005, Imaging microglia in live brain slices and slice cultures, In: *Imaging in Neuroscience and Development* (R. Yuste and A. Konnerth, eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Lakowicz, J.R., Szmacinski, H., Nowaczyk, K., Berndt, K.W., and Johnson, M., 1992, Fluorescence lifetime imaging, *Anal. Biochem*. 202:316–330.

Lansford, R., Bearman, G., and Fraser, S.E., 2001, Resolution of multiple green fluorescent protein color variants and dyes using two-photon microscopy and imaging spectroscopy, *J. Biomed. Opt*. 6:311–318.

Lawson, N.D., and Weinstein, B.M., 2002, *In vivo* imaging of embryonic vascular development using transgenic zebrafish, *Dev. Biol*. 248:307–318.

Legg, J.W., Lewis, C.A., Parsons, M., Ng, T., Isacke, C.M., 2002, A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility, *Nat. Cell Biol.* 4(6):399–407.

Lepe-Zuniga, J.L., Zigler, J.S. Jr., and Gery, I., 1987, Toxicity of light-exposed Hepes media, *J. Immunol. Methods* 103:145.

Lin, H.J., Herman, P., and Lakowicz, J.R., 2003, Fluorescence lifetime-resolved pH imaging of living cells, *Cytometry* 52A:77–89.

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A., 2001, Studying protein dynamics in living cells, *Nat. Rev. Mol. Cell. Biol*. 2:444–456.

Lippincott-Schwartz, J., Altan-Bonnet, N., and Patterson, G.H., 2003, Review: Photobleaching and photoactivation: Following protein dynamics in living cells, *Nat. Cell Biol*. 5:S7–S14.

Loew, L.M., 1993, Confocal microscopy of potentiometric fluorescent dyes, *Methods Cell Biol*. 38:195–209.

Lorenzl, S., Koedel, U., Dirnagl, U., Ruckdeschel, G., and Pfister, H.W., 1993, Imaging of leukocyte-endothelium interaction using *in vivo* confocal laser scanning microscopy during the early phase of experimental pneumococcal meningitis, *J. Infect. Dis*. 168:927–933.

Lucius, R., Mentlein, R., and Sievers, J., 1998, Riboflavin-mediated axonal degeneration of postnatal retinal ganglion cells *in vitro* is related to the formation of free radicals, *Free Radic. Biol. Med*. 24:798–808.

Magde, D., Elson, E.L., and Webb, W.W., 1974, Fluorescence correlation spectroscopy. II. An experimental realization, *Biopolymers* 13:29–61.

Manders, E.M.M., Stap, J., Strackee, J., Van Driel, R., and Aten, J.A., 1996, Dynamic behavior of DNA replication domains, *Exp. Cell Res*. 226:328–335.

Manders, E.M.M., Kimura, H., and Cook, P.R., 1999, Direct imaging of DNA in living cells reveals the dynamics of chromosome formation, *J. Cell Biol*. 144:813–821.

Manders, E.M.M., Visser, A.E., Koppen, A., de Leeuw, W.C., van Liere, R., Brakenhoff, G.J., and van Driel, R., 2003, Four-dimensional imaging of chromatin dynamics during the assembly of the interphase nucleus, *Chromosome Res*. 11:537–547.

Marrs, G.S., Green, S.H., and Dailey, M.E., 2001, Rapid formation and remodeling of postsynaptic densities in developing dendrites, *Nat. Neurosci*. 4:1006–1013.

Masters, B.R., 1992, Confocal microscopy of the in-situ crystalline lens, *J. Microsc*. 165:159–167.

McKenna, N., and Wang, Y.L., 1986, Culturing cells on the microscope stage, *Methods Cell Biol*. 29:195–205.

Megason, S.G., and Fraser, S.E., 2003, Digitizing life at the level of the cell: High-performance laser-scanning microscopy and image analysis for in toto imaging of development, *Mechan. Dev*. 120:1407–1420.

Mehta, A.D., Jung, J.C., Blusberg, B.A., and Schnitzer, M.J., 2004, Fiber-optic *in vivo* imaging in the mammalian nervous system, *Curr. Opin. Neurobiol*. 14:617–628.

Meyvis, T.K., De Smedt, S.C., Van Oostveldt, P., and Demeester, J., 1999, Fluorescence recovery after photobleaching: A versatile tool for mobility and interaction measurements in pharmaceutical research, *Pharm. Res*. 16: 1153–1162.

Miller, M.J., Wei, S.H., Parker, I., and Cahalan, M.D., 2002, Two-photon imaging of lymphocyte motility and antigen response in intact lymph node, *Science* 296:1869–1973.

Miller, M.J., Safrina, O., Parker, I., and Cahalan, M.D., 2004, Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes, *J. Exp. Med*. 200:847–856.

Minta, A., Kao, J., and Tsien, R., 1989, Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores, *J. Biol Chem*. 264:8171–8178.

Miyawaki, A., 2003, Fluorescence imaging of physiological activity in complex systems using GFP-based probes, *Curr. Opin. Neurobiol*. 13:591–596.

Mizrahi, A., Crowley, J.C., Shtoyerman, E., and Katz, L.C., 2004, High-resolution *in vivo* imaging of hippocampal dendrites and spines, *J. Neurosci*. 24:3147–3151.

Moné, M.J., Bernas, T., Dinant, C., Goedvree, F.A., Manders, E.M.M., Volker, M., Houtsmuller, A.B., Hoeijmakers, J.H.J., Vermeulen, W., and van Driel, R., 2004, *In vivo* dynamics of chromatin-associated complex formation in mammalian nucleotide excision repair, *Proc. Natl. Acad. Sci. USA* 101:15933–15937.

Montoya, M.C., Sancho, D., Bonello, G., Collette, P., Langlet, C., He, H.T., Aparicio, P., Alcover, A., Olive, D., and Sanchez-Madrid, F., 2002, Role of ICAM-3 in the initial interaction of T lymphocytes and APCs, *Nat. Immunol*. 3:159–168.

Mulligan, S.J., MacVicar, B.A., 2004, Calcium transients in astrocyte endfeet cause cerebrovascular constrictions, *Nature* 431(7005):195–199.

Myrdal, S., and Foster, M., 1994, Time-resolved confocal analysis of antibody penetration into living, solid tumor spheroids, *Scanning* 16:155–167.

Nehls, S., Snapp, E.L., Cole, N.B., Zaal, K.J., Kenworthy, A.K., Robert, T.H., Ellenberg, J., Presley, J.F., Siggia, E., and Lippincott-Schwartz, J., 2000, Dynamics and retention of misfolded proteins in native ER membranes, *Nat. Cell Biol*. 2:288–295.

Niell, C.M., Meyer, M.P., and Smith, S.J., 2004, *In vivo* imaging of synapse formation on a growing dendritic arbor, *Nat. Neurosci*. 7:254–260.

Niggli, E., and Egger, M., 2004, Applications of multi-photon microscopy in cell physiology, *Front Biosci*. 9:1598–1610.

Noctor, S.C., Martinez-Cerdeno, V., Ivic, L., and Kriegstein, A.R., 2004, Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases, *Nat. Neurosci*. 7:136–144.

O'Malley, D.M., Kao, Y.H., and Fetcho, J.R., 1996, Imaging the functional organization of zebrafish hindbrain segments during escape behaviors, *Neuron* 17:1145–1155.

O'Rourke, N.A., and Fraser, S.E., 1990, Dynamic changes in optic fiber terminal arbors lead to retinotopic map formation: An *in vivo* confocal microscopic study, *Neuron* 5:159–171.

O'Rourke, N.A., Dailey, M.E., Smith, S.J., and McConnell, S.K., 1992, Diverse migratory pathways in the developing cerebral cortex, *Science* 258:299–302.

Paddock, S.W., 2002, Confocal imaging of *Drosophila* embryos, *Methods Cell Biol*. 70:361–378.

Pagano, R.E., Martin, O.C., Kang, H.C., and Haugland, R.P., 1991, A novel fluorescent ceramide analogue for studying membrane traffic in animal cells: Accumulation at the Golgi apparatus results in altered spectral properties of the sphingoid precursor, *J. Cell Biol*. 11:1267–1279.

Park, M.K., Tepikin, A.V., and Petersen, O.H., 2002, What can we learn about cell signalling by combining optical imaging and patch clamp techniques? *Pflugers Arch*. 444:305–316.

Pasti, L., Zonta, M., Pozzan, T., Vicini, S., and Carmignoto, G., 2001, Cytosolic calcium oscillations in astrocytes may regulate exocytotic release of glutamate, *J. Neurosci*. 21:477–484.

Patterson, G.H., and Lippincott-Schwartz, J., 2002, A photoactivatable GFP for selective photolabeling of proteins and cells, *Science* 297:1873–1877.

Patterson, G.H., and Lippincott-Schwartz, J., 2004, Selective photolabeling of proteins using photoactivatable GFP, *Methods* 32:445–450.

Pepperkok, R., Squire, A., Geley, S., and Bastiaens, P.I., 1999, Simultaneous detection of multiple green fluorescent proteins in live cells by fluorescence lifetime imaging microscopy, *Curr. Biol*. 9:269–272.

Periasamy, A., and Day, R.N., 1999, Visualizing protein interactions in living cells using digitized GFP imaging and FRET microscopy, *Methods Cell Biol*. 58:293–314.

Periasamy, A., Elangovan, M., Elliott, E., and Brautigan, D.L., 2002, Fluorescence lifetime imaging (FLIM) of green fluorescent fusion proteins in living cells, *Methods Mol. Biol*. 183:89–100.

Peter, M., Ameer-Beg, S.M., 2004, Imaging molecular interactions by multiphoton FLIM, *Biol. Cell.* 96(3):231–236.

Petersen, M., and Dailey, M.E., 2004, Diverse microglial motility behaviors during clearance of dead cells in hippocampal slices, *Glia* 46:195–206.

Petran, M., Hadravsky, M., Benes, J., and Boyde, A., 1986, *In vivo* microscopy using the tandem scanning microscope, *Ann. N.Y. Acad. Sci*. 483:440–447.

Petroll, W.M., Cavanagh, H.D., Lemp, M.A., Andrews, P.M., and Jester, J.V., 1992, Digital image acquisition in *in vivo* confocal microscopy, *J. Microsc*. 165:61–69.

Petroll, W.M., Cavanagh, H.D., and Jester, J.V., 1993, Three-dimensional imaging of corneal cells using *in vivo* confocal microscopy, *J. Microsc*. 170:213–219.

Politz, J.C., 1999, Use of caged fluorochromes to track macromolecular movement in living cells, *Trends Cell Biol*. 9:284–287.

Pologruto, T.A., Yasuda, R., and Svoboda, K., 2004, Monitoring neural activity and [Ca^{2+}] with genetically encoded Ca^{2+} indicators, *J. Neurosci*. 24:9572–9579.

Poole, C.A., Brookes, N.H., and Clover, G.M., 1993, Keratocyte networks visualized in the living cornea using vital dyes, *J. Cell Sci*. 106:685–692.

Potter, S. M., 2004. Two-photon microscopy for 4D imaging of living neurons, In: *Imaging in Neuroscience and Development: A Laboratory Manual*, 2nd ed., (R. Yuste and A. Konnerth, eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 8.1–8.12.

Potter, S.M., and DeMarse, T.B., 2001, A new approach to neural cell culture for long-term studies, *J. Neurosci. Methods* 110:17–24.

Reits, E.A., and Neefjes, J.J., 2001, From fixed to FRAP: measuring protein mobility and activity in living cells. *Nat. Cell Biol*. 3(6):E145–E147.

Rios, E., Shirokova, N., Kirsch, W.G., Pizarro, G., Stern, M.D., Cheng, H., and Gonzalez, A., 2001, A preferred amplitude of calcium sparks in skeletal muscle, *Biophys. J*. 80:169–183.

Robb, D.L., and Wylie, C., 1999, Confocal microscopy on *Xenopus laevis* oocytes and embryos, *Methods Mol. Biol*. 122:173–183.

Rubart, M., 2004, Two-photon microscopy of cells and tissue, *Circ. Res*. 95:1154–1166.

Salmon, W.C., Adams, M.C., and Waterman-Storer, C.M., 2002, Dual-wavelength fluorescent speckle microscopy reveals coupling of microtubule and actin movements in migrating cells, *J. Cell Biol*. 158:31–37.

Schmidt, C.E., Horwitz, A.F., Lauffenburger, D.A., and Sheetz, M.P., 1993, Integrin-cytoskeletal interactions in migrating fibroblasts are dynamic, asymmetric, and regulated, *J. Cell Biol*. 123:977–991.

Schwarzbauer, J.E., 1997, Cell migration: may the force be with you, *Curr. Biol.* 7(5):R292–R294.

Sekar, R.B., and Periasamy, A., 2003, Fluorescence resonance energy transfer (FRET) microscopy imaging of live-cell protein localizations, *J. Cell Biol*. 160:629–633.

Seyfried, V., Birk, H., Storz, R., and Ulrich, H., 2003, Advances in multispectral confocal imaging, *Progress in Biomedical Optics and Imaging* 5139:146–157.

Sheetz, M.P., ed., 1998, *Laser Tweezers in Cell Biology*, Academic Press, San Diego, California.

Siegel, W.H., and Pritchett, T., 2000, Tutorial: Examining the relationship between media and light, *Biopharmaceuticals* 13:65–66.

Silva, E., Salim-Hanna, M., Edwards, A.M., Becker, M.I., and De Ioannes, A.E., 1991, A light-induced tryptophan-riboflavin binding: biological implications, *Adv. Exp. Med. Biol*. 289:33–48.

Silva, E., and Godoy, J., 1994, Riboflavin sensitized photooxidation of tyrosine, *Int. J. Vitam. Nutr. Res*. 64:253–256.

Smith, S.J., Cooper, M., and Waxman, A., 1990, Laser microscopy of subcellular structure in living neocortex: Can one see dendritic spines twitch? In: *XYIII Symposia Medica Hoechst, Biology of Memory* (L. Squire and E. Lindenlaub, eds.), Schattauer, Stuttgart, Germany, pp. 49–71.

Soll, D., 1995, The use of computers in understanding how animal cells crawl, *Int. Rev. Cytol*. 163:43–104.

Soll, D.R., 1999, Computer-assisted three-dimensional reconstruction and motion analysis of living, crawling cells, *Comput. Med. Imaging Graphics* 23:3–14.

Spierenburg, G.T., Oerlemans, F.T., van Laarhoven, J.P., and de Bruyn, C.H., 1984, Phototoxicity of N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid-buffered culture media for human leukemic cell lines, *Cancer Res*. 44:2253–2254.

Stence, N., Waite, M., and Dailey, M.E., 2001, Dynamics of microglial activation: A confocal time-lapse analysis in hippocampal slices, *Glia* 33:256–266.

Stoll, S., Delon, J., Brotz, T.M., and Germain, R.N., 2002, Dynamic imaging of T cell-dendritic cell interactions in lymph nodes, *Science* 296:1873–1876.

Stoppini, L., Buchs, P.A., and Muller, D., 1991, A simple method for organotypic cultures of nervous tissue, *J. Neurosci. Methods* 37:173–182.

Strange, K., and Spring, K.R., 1986, Methods for imaging renal tubule cells, *Kidney Int*. 30:192–200.

Streit, W.J., and Kreutzberg, G.W., 1987, Lectin binding by resting and reactive microglia, *J. Neurocytol*. 16:249–260.

Stricker, S.A., 2004, Dual-channel confocal ratioing of calcium dynamics in living eggs and oocytes, *Methods Mol. Biol*. 254:137–148.

Stryer, L., 1978, Fluorescence energy transfer as a spectroscopic ruler, *Annu. Rev. Biochem*. 47:819–846.

Sullivan, W., Daily, D.R., Fogarty, P., Yook, K.J., and Pimpinelli, S., 1993, Delays in anaphase initiation occur in individual nuclei of the syncytial *Drosophila* embryo, *Mol. Biol. Cell* 4:885–896.

Svoboda, K., Denk, W., Kleinfeld, D., and Tank, D., 1997, *In vivo* dendritic calcium dynamics in neocortical pyramidal neurons, *Nature* 385:161–165.

Tauer, U., 2002, Advantages and risks of multiphoton microscopy in physiology, *Exp. Physiol*. 87:709–714.

Terasaki, M., Song, J., Wong, J.R., Weiss, M.J., and Chen, L.B., 1984, Localization of endoplasmic reticulum in living and glutaraldehyde fixed cells with fluorescent dyes, *Cell* 8:101–108.

Thomas, C.F., and White, J.G., 1998, Four-dimensional imaging: The exploration of space and time, *Trends Biotechnol*. 16:175–182.

Thompson, N.L., 1991, Fluorescence correlation spectroscopy, In: *Topics in Fluorescence Spectroscopy*, Vol. 1 (J. R. Lakowicz, ed.), Plenum Press, New York, pp. 337–378.

Trachtenberg, J.T., Chen, B.E., Knott, G.W., Feng, G., Sanes, J.R., Welker, E., and Svoboda, K., 2002, Long-term *in vivo* imaging of experience-dependent synaptic plasticity in adult cortex, *Nature* 420:788–794.

Tsurui, H., Nishimura, H., Hattori, S., Hirose, S., Okumura, K., and Shirai, T., 2000, Seven-color fluorescence imaging of tissue samples based on Fourier spectroscopy and singular value decomposition, *J. Histochem. Cytochem*. 48:653–662.

Verschure, P.J., van der Kraan, I., Manders, E.M., and van Driel, R., 1999, Spatial relationship between transcription sites and chromosome territories, *J. Cell Biol*. 147:13–24.

Vesely, P., Maly, J., Cumpelik, J., Pluta, M., and Tuma V., 1982, Improved spatial and temporal resolution in an apparatus for time-lapse, phase contrast cine light micrography of cells *in vitro*, *J. Microsc*. 125:67–76.

Visscher, K., and Brakenhoff, G.J., 1991, Single beam optical trapping integrated in a confocal microscope for biological applications, *Cytometry* 12:486–491.

Walcerz, D.B., and Diller, K.R., 1991, Quantitative light microscopy of combined perfusion and freezing processes, *J. Microsc*. 161:297–311, and US Patent 5,257,128.

Wang, S.Q., Wei, C., Zhao, G., Brochet, D.X., Shen, J., Song, L.S., Wang, W., Yang, D., and Cheng, H., 2004, Imaging microdomain Ca^{2+} in muscle cells, *Circ. Res*. 94:1011–1022.

Waterman-Storer, C.M., Sanger, J.W., and Sanger, J.M., 1993, Dynamics of organelles in the mitotic spindles of living cells: Membrane and microtubule interactions, *Cell Motil. Cytosketelon* 26:19–39.

Waterman-Storer, C.M., Desai, A., Bulinski, J.C., and Salmon, E.D., 1998, Fluorescent speckle microscopy, a method to visualize the dynamics of protein assemblies in living cells, *Curr. Biol*. 8:1227–1230.

Weiss, M., 2004, Challenges and artifacts in quantitative photobleaching experiments, *Traffic* 5:662–671.

Wiseman, P.W., and Petersen, N.O., 1999, Image correlation spectroscopy. II. Optimization for ultrasensitive detection of preexisting platelet-derived growth factor-beta receptor oligomers on intact cells, *Biophys. J*. 76:963–977.

Wiseman, P.W., Squier, J.A., Ellisman, M.H., and Wilson, K.R., 2000, Two-photon image correlation spectroscopy and image cross-correlation spectroscopy, *J. Microsc*. 200:14–25.

Wood, C., Kabat, E.A., Murphy, L.A., and Goldstein, I.J., 1979, Immunochemical studies of the combining sites of the two isolectins, A4 and B4, isolated from Bandeiraea simplicifolia, *Arch. Biochem. Biophys*. 198:1–11.

Wouters, F.S., Verveer, P.J., and Bastiaens, P.I.H., 2001, Imaging biochemistry inside cells, *Trends Cell Biol.* 11:203–211.

Yoder, E.J., and Kleinfeld, D., 2002, Cortical imaging through the intact mouse skull using two-photon excitation laser scanning microscopy, *Microsc. Res. Techn*. 56:304–305.

Zhang, S., Boyd, J., Delaney, K., Murphy, T.H., 2005, Rapid reversible changes in dendritic spine structure in vivo gated by the degree of ischemia, *J. Neurosci.* 25(22):5333–5338.

Zicha, D., Dobbie, I.M., Holt, M.R., Monypenny, J., Soong, D.Y., Gray, C., and Dunn, G.A., 2003, Rapid actin transport during cell protrusion, *Science* 300:142–145.

Zieger, M.A., Glofcheski, D.J., Lepock, J.R., and Kruuv, J., 1991, Factors influencing survival of mammalian cells exposed to hypothermia. V. Effects of hepes, free radicals, and H_2O_2 under light and dark conditions, *Cryobiology* 28:8–17.

Zigler, J.S. Jr., Lepe-Zuniga, J.L., Vistica, B., and Gery, I., 1985, Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium, In Vitro *Cell Dev. Biol*. 21:282–287.

Zimmermann, T., Rietdorf, J., Girod, A., Georget, V., and Pepperkok, R., 2002, Spectral imaging and linear un-mixing enables improved FRET efficiency with a novel GFP2-YFP FRET pair, *FEBS Lett*. 531:245–249.

Zimmermann, T., Rietdorf, J., and Pepperkok, R., 2003, Spectral imaging and its applications in live-cell microscopy, *FEBS Lett*. 546:87–92.

Zuo, Y., Lubischer, J.L., Kang, H., Tian, L., Mikesh, M., Marks, A., Scofield, V.L., Maika, S., Newman, C., Krieg, P., and Thompson, W.J., 2004. Fluorescent proteins expressed in mouse transgenic lines mark subsets of glia, neurons, macrophages, and dendritic cells for vital examination, *J. Neurosci*. 24:10999–11009.