Light Microscopy Techniques for Live Cell Imaging

David J. Stephens[1] and Victoria J. Allan[2]*

Since the earliest examination of cellular structures, biologists have been fascinated by observing cells using light microscopy. The advent of fluorescent labeling technologies plus the plethora of sophisticated light microscope techniques now available make studying dynamic processes in living cells almost commonplace. For anyone new to this area, however, it can be daunting to decide which techniques or equipment to try. Here, we aim to give a brief overview of the main approaches to live cell imaging, with some mention of their pros and cons.

We have an incredibly detailed view of how proteins and lipids interact inside cells to govern the generation, maintenance, and function of cellular organization, as determined from biochemical and genetic experiments spanning diverse approaches from in vitro reconstitution of cellular processes to atomic resolution structure determination. However, these techniques only provide a static, snapshot view of cells. Being able to observe processes as they happen within the cell by light microscopy adds a vital extra dimension to our understanding of cell function. Perhaps the commonest approach for studying dynamic cellular events is live cell fluorescence microscopy, and we will discuss this in some detail. However, transmitted light techniques also have an important part to play (*1*) and not just as an adjunct to fluorescence imaging.

Environmental considerations. Regardless of the imaging technique to be used, it is crucial to consider the cells' health on the microscope stage. Cells are sensitive to photodamage, particularly in the presence of fluorophores (which generate free radicals upon photobleaching), and there are many ways of trying to limit light-induced damage. It is also vital to keep the cellular environment constant. There are a number of solutions to this problem, including the control of temperature, humidity, and CO_2. Environmental control ranges from simple heating jackets to Perspex boxes that fully encase a system. The relative importance of each parameter will vary between samples, but the overriding concern for all three is stability. In time-lapse experiments, once a sample is being imaged, the focal plane must remain stable. Autofocus routines are available, which can compensate for focus drift to some extent, but they require additional illumination of the sample. One important, but often overlooked, cause of focus drift is air conditioning units, which can cause cyclic changes in focus as they turn on and off.

Fluorescence Imaging

Whilst it is sometimes possible to image endogenous cellular molecules such as NAD(P)H (*2*) by their inherent fluorescence, it is far more common to introduce exogenous fluorescent molecules. The advent of green fluorescent protein (GFP) technology has revolutionized live cell imaging because an autofluorescent molecule can be genetically encoded as a fusion with the cDNA of interest (*3*). The spectral variants of GFP and the unrelated red fluorescent protein (*4*) make it feasible to perform multicolor imaging of living cells. The simultaneous study of multiple fluorophores or ratiometric analysis of a single probe requires spectral separation of both the excitation and emission light. Commercial systems are now available for "spectral unmixing" of data, and this allows the use of closely related fluorophores, but, where possible, it is better to use probes with distinct excitation and emission spectra that are separable at the point of image acquisition.

GFP-based biosensors are opening many fields to optical techniques, notably the spatio-temporal analysis of signaling events following the development of probes for diverse processes including heterotrimeric G protein activity (*5*) and phosphoinositide signaling using GFP-tagged pleckstrin homology (PH) domain constructs (*6*). The field of calcium imaging also makes use of GFP-based probes (*7*), allowing organelle-specific analysis of calcium dynamics. GFP-tagging is also being applied to high-throughput analyses to provide further functional annotation of genome sequences (*8*).

FlAsH (fluorescent arsenical helix binder) labeling provides another means for fluorescent labeling of genetically encoded probes (*9*). It is mediated by engineering a tetracysteine motif into the target protein and then incubating cells with a nonfluorescent biarsenical compound that becomes strongly fluorescent upon binding to this tetracysteine motif. A recent development enables multicolor labeling and photoconversion of diaminobenzidine for correlative electron microscopy (*10*). Unfortunately FlAsH compounds can also label endogenous proteins containing similar tetracysteine motifs (*11*). In addition, the cysteine residues must be in the reduced state for labeling to occur, and antidotes must be added simultaneously with labeling to avoid toxicity problems.

There are, of course, many other potential probes that can be introduced into cells. Specific fluorescent lipid molecules and organelle-specific dyes are often cell permeable and can simply be added to the culture medium (*12*). Fluorescently labeled proteins can be introduced by microinjection, and the usefulness of such probes is also continually driving the technology for studying intracellular dynamics. A prime example of this is the development of fluorescence speckle microscopy (*13*). Here, introduction of a limited amount of fluorescent protein to a polymeric structure, such as a microtubule or actin filament, results in a "speckled" appearance. These speckles can then be imaged over time and tracked within the cell to provide accurate quantitative analysis of polymer dynamics. Finally, a number of probes can be activated by light, allowing specific detection only after a pulse of illumination (*14–16*).

Live Cell Imaging

When selecting which system to use for imaging living cells, one should consider three things: sensitivity of detection, speed of acquisition, and the viability of the specimen. Light microscopy of living versus fixed samples is essentially a trade-off between acquiring images with a high signal-to-noise ratio and damaging the sample under observation; this is a particularly critical issue in live cell imaging. Other important questions center on the sample you want to image. Is it thick or thin? Is the process to be observed fast or slow? Do you need to image for seconds, minutes, hours, or days, and at how many different wavelengths does the image need to be sampled? How bright is your signal? You also need to consider several further ques-

[1]Department of Biochemistry, University of Bristol, School of Medical Sciences, University Walk, Bristol, BS8 1TD, UK. [2]School of Biological Sciences, Oxford Road, University of Manchester, Manchester, M13 9PT, UK.

*To whom correspondence should be addressed. E-mail: viki.allan@man.ac.uk

tions: Will you want to use a specialized technique such as photobleaching? Are transmitted light images required, and if so, of what quality? In many cases, no single microscope system will be best, and compromises will have to be made. A good understanding of the pros and cons of different microscopes is needed, and it is also helpful to understand the resolution of the light microscope (*17*). The basic features of three types of fluorescence microscope systems are illustrated in Fig. 1.

Limiting cell damage. Because illumination of fluorophores causes photobleaching and therefore cell damage, everything possible should be done to limit the duration and intensity of illumination. A minimum requirement is the ability to shut off illumination light when it is not needed; this is inherent in confocal systems and can easily be achieved for widefield systems that use electronic shutters controlled by computer (which will usually control the image acquisition as well). Care should also be taken to remove unwanted wavelengths of light and not to rely simply on the excitation filters. Reducing the level of oxygen can help reduce photobleaching and free radical production. Oxygen can be removed from the medium as long as the cells are in a sealed chamber (*13*), providing the cells tolerate oxygen withdrawal. Finally, omitting phenol red and serum from the medium (again, if your cells will stand it) will help reduce background fluorescence.

The system must also make best possible use of the light, so high numerical aperture objectives should be used, and there should be as few optical elements in the light path as possible. The sensitivity of the camera (or photomultiplier tube, if using a confocal) will be vital (*18*), because the more sensitive the detector, the lower the illumination intensity needed. Using an intensified camera is one way of increasing sensitivity, at the expense of increasing noise in the image. Alternatively, sensitive back-illuminated charge-coupled device (CCD) cameras with thinned chips are available. A new type of camera that amplifies the CCD readout signal on the chip (*19*) offers further possible advantages. Another simple way of increasing camera sensitivity is to combine signals from multiple pixels (called binning), although this process reduces image resolution.

Speed of acquisition. A key consideration is speed of data acquisition, particularly when multiple fluorophores are imaged simultaneously or when a single probe is analyzed ratiometrically. Switching between laser lines, filters, or output from a monochromator will slow data acquisition (Fig. 1). Monochromator-based systems have the advantage of rapid switching between excitation wavelengths (typically <3 ms) but suffer from reduced illumination intensity, principally due to fiber optic coupling to the microscope. Filter wheel configurations usually have higher light throughput but are often slower in switching. Data acquisition rates of conventional scanning confocal microscopes are fast enough for rapid imaging if only small regions are sampled. To image very fast processes such as neuronal network activity, one may obtain faster scanning by using resonant galvanometers (which are optional on many commercial systems). Another important consideration is that scanning systems acquire data pixel by pixel, whereas CCD cameras acquire a whole field of view at once.

Scanning speed in confocal microscopy can also be improved with the use of multifocal imaging (*20*). Here, the excitation light

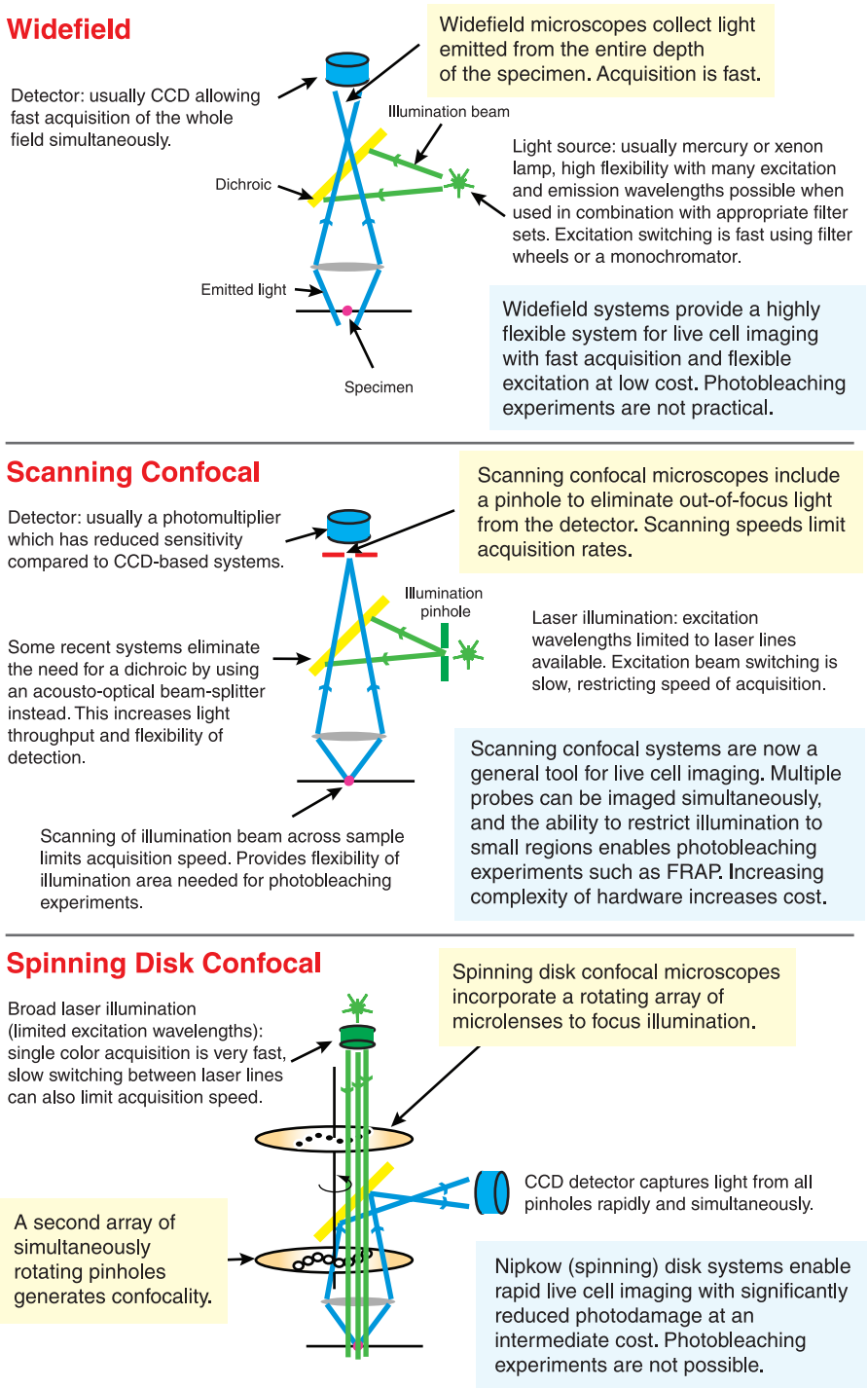

Fig. 1. Comparison of widefield, scanning confocal, and spinning disk confocal systems, with schematics of each. All systems are capable of being equipped for 3D and 4D data acquisition. Excitation beams are shown in green; emission beams, in blue. The differences between these systems mean that no single system is suited for every experiment. Typical system configurations are shown, and user modification and options allow great flexibility.

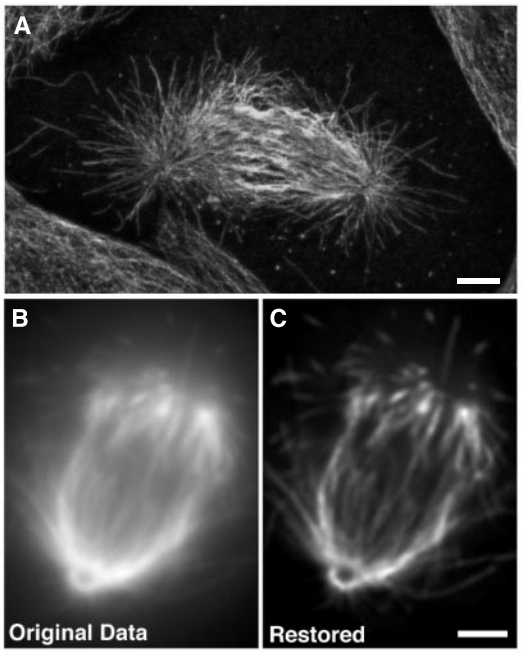

Fig. 2. Examples of 3D images obtained by confocal and widefield deconvolution microscopy. (**A**) A mitotic spindle in a DLD1 cell imaged by single photon confocal microscopy (*61*). (**B** and **C**) A mitotic spindle in a *Xenopus* XLK2 cell imaged by 3D widefield microscopy [adapted from (*21*), with permission from Eaton Publishing]. A single plane of a *z* series without additional processing [(B), original data] and the same data set after restoration by constrained iterative deconvolution [(C), restored] are shown. Scale bar, 2 μm.

beam is split into multiple foci from which data are collected simultaneously with a CCD (Fig. 1). Nipkow disk confocal microscopy is available commercially through a number of suppliers and can achieve speeds of 360 frames per second. Sensitivity and data acquisition rates of Nipkow disk systems, like widefield microscopes, depend on the quality and the readout time of the detector CCD. No single camera will perform optimally for all tasks, and correct matching of optics and electronics is essential for best performance.

Three- and four-dimensional (3- and 4D) imaging. Researchers are often attracted to confocal systems because high-resolution 3D images (Fig. 2) can be acquired simply. However, many experiments, particularly those using live cells, may be better performed using widefield (conventional) systems with subsequent deconvolution of the data series. Widefield microscopes do not exclude light from any plane of focus; they collect it all. The contribution of light from an infinitely small point source to a plane of focus some distance away from that point source is described by the point spread function (PSF) of the objective. Determination of the PSF of a system enables mathematical reassignment of the out-of-focus light back to its point source by deconvolution (*21*). This approach has been used with great success in both cell and developmental biology, and it can be particularly advantageous in imaging very weakly fluorescent structures such as microtubules (Fig. 2) (*22*). Deconvolution must be applied with great care and accuracy, however, to avoid the generation of artefacts (*21*). Deconvolution of large 4D data series can now be achieved in minutes to hours with the use of dual processor personal computers.

Most cellular processes occur in three dimensions over time, so to get a complete picture we need to image cells in four dimensions. Most confocal systems and epi-illumination microscopes are either provided with or can be simply adapted to include a means of acquiring data series in four dimensions. Perhaps the most important consideration is the speed, accuracy, and reproducibility of the *z* position change. Piezoelectric objective drives have the edge here, enabling high-speed acquisition of stacks at multiple wavelengths over time. Multidimensional live cell imaging also requires tools for data analysis (*23*). Tools are continually being developed for particle tracking of objects moving inside cells such as transport vesicles (*24*). Most reconstruction approaches assume a uniform refractive index through the sample, which is not encountered often in reality (*25*); further developments are needed to address this issue.

Multiphoton approaches to in vivo imaging. Multiphoton confocal systems are now available from several companies. The two-photon effect excites a chromophore not by a single photon but from two photons being absorbed within a femtosecond time scale (*26*). This enables the use of longer wavelength excitation, which penetrates deeper into samples and reduces photobleaching. Notably, the analysis of intact organisms or tissues greatly benefits from this technique, allowing imaging in situ (*27*). Such systems have been used for imaging both tumor development (*28*) and the pathophysiology of Alzheimer's (*29*) by replacing a small part of the skull with a coverslip. Alternatively, imaging of neuronal processes through thinned skulls is also possible (*27*). However, other techniques, using single photon excitation, have also been applied with great success to the imaging of protein interactions (*30*) and the analysis of gene expression (*31*) in living animals. These approaches are likely to be developed toward medically applicable systems for diagnosis and treatment of patients, extending the capabilities of existing magnetic resonance imaging and positron emission tomography technologies. Optical imaging is likely to be of great benefit to the application of gene therapy in combination with nontoxic fluorescent reporters and of monitoring cancer progression and treatment. Similarly, confocal imaging has recently been coupled with endoscopy (*32*) with diagnostic potential.

Other Imaging Modes

Bright-field imaging. Imaging living cells with transmitted light is often used along with fluorescence microscopy in order to provide information on cell shape, position, and motility. This is absolutely vital when studying processes such as apoptosis and mitosis, where cells undergo drastic shape changes. Phase contrast and differential interference contrast (DIC, also called Normarski) microscopy are the most commonly used. To switch between transmitted

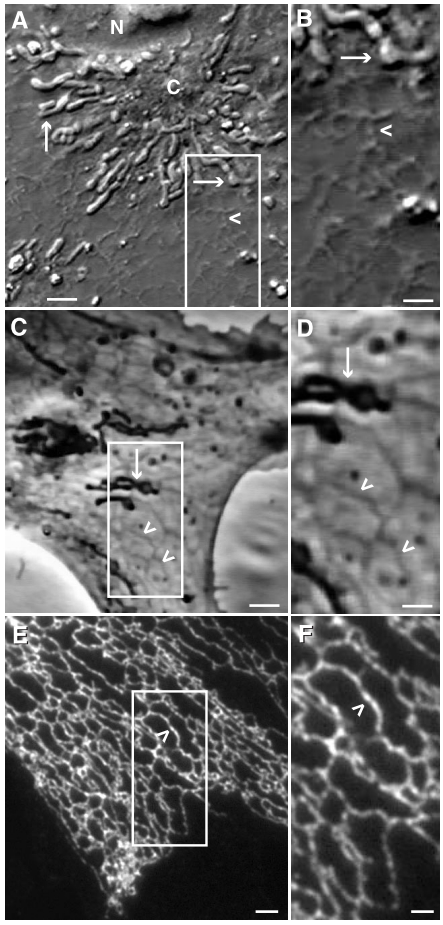

Fig. 3. Video-enhanced transmitted light microscopy. Imaging living cells by video-enhanced differential interference contrast (**A** and **B**) and phase contrast (**C** and **D**) microscopy reveals a wealth of information on organelles including mitochondria (arrows) and the endoplasmic reticulum (ER) (arrowheads). The nucleus (N) and centrosomal area (C) are marked in (A). The ER is more obvious in the associated movie clips of a Vero cell [(A) and (B), from movie S1] and a *Xenopus* tissue culture (XTC) cell [(C) and (D), from movie S2], imaged as described (*61*). An immunofluorescence image of the ER in an XTC cell (*61*) is shown for comparison (**E** and **F**). (B), (D), and (F) are enlargements of the boxed areas in (A), (C), and (E). Scale bars in (A), (C), and (E), 2 μm ; in (B), (D), and (F), 1 μm.

and fluorescence imaging under computer control requires a shutter on the transmitted light path in addition to a fluorescence shutter. A complication with DIC is that it needs a polarizing filter (the analyzer) between the objective and the camera, and if this is left in place during fluorescence image capture it will reduce the intensity of the image reaching the camera. There are some systems available to avoid this, which have a motorized analyzer that can be moved out of the light path, or an analyzer that works only at certain wavelengths of light.

Whilst a simple image captured by the microscope camera system will be enough for many experiments, there is huge potential for obtaining detailed insight into cell function by pushing transmitted systems to the limits by using the best possible optics and specialized image-processing equipment (*33*). This is clearly illustrated in Fig. 3 and associated movies S1 and S2 [see also (*1*)]. In addition, there are other specialized light microscopy techniques, such as reflection contrast microscopy (*34*) and DRIMAPS [Digitally Recorded Interference Microscopy with Automatic Phase Shifting, (*35*)], that provide different types of information about cell structure and function. However, all of these advanced transmitted light techniques do require specialist equipment and knowledge, which probably explains their rather limited use at present. In addition, researchers will often only be interested in a single organelle or structure, which will make fluorescence the method of choice.

Total internal reflection. Many cellular processes occur in specifically restricted areas of the cell, such as the plasma membrane. Total internal reflection fluorescence microscopy (TIRFM) (Fig. 4) provides a means of direct imaging of processes within very close proximity to the coverslip (*36*). Excitation at a critical angle generates an evanescent field of excitation light that decays rapidly with distance from the coverslip, limiting the depth of excitation to a distance of ~100 nm. TIRFM of live cells has given insight into the role of actin and dynamin in endocytosis (*37*) and can also be combined with other techniques such as photobleaching (*38*) or widefield imaging. One of the most exciting recent developments has been the ability to image single molecules in living cells (*39*), and examples of this include growth factor receptor signaling (*40*) and viral infection (*41*). Although technically demanding and requiring state-of-the-art equipment, the coupling of these technologies with GFP-based expression strategies is sure to lead to further developments in the near future. Systems for TIRFM are now commercially available but, as with most of the recent developments in microscopy, require skilled use and careful interpretation of data.

Fluorescence correlation spectroscopy. Cellular processes can also be imaged using a very small area of illumination by fluorescence correlation spectroscopy (FCS), which is used to quantitatively measure local concentration and diffusion of particles through a very small volume and can now be applied to live cells (*42*). Due to its high sensitivity, the technique is prone to imaging artefacts such as intramolecular changes in fluorescence, including photobleaching. However, a key advantage of FCS is that it is also applicable to single molecule studies.

Photobleaching and photoactivation approaches. Because scanning confocal microscopy has control over the region of illumination, it is ideal for photobleaching techniques such as fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) (*43*, *44*). Both are now widely used to measure diffusional mobility of GFP-tagged proteins in cells. Increasingly, they are combined with kinetic modeling of cellular processes (*45*) to study topics as diverse as membrane traffic and nuclear architecture and function (*46*). Probes that can be light-activated such as photoactivatable-GFP and Kaede (*14*), both of which show greatly enhanced fluorescence emission following activation at ~400 nm, allow selective labeling of subdomains of cells and organelles.

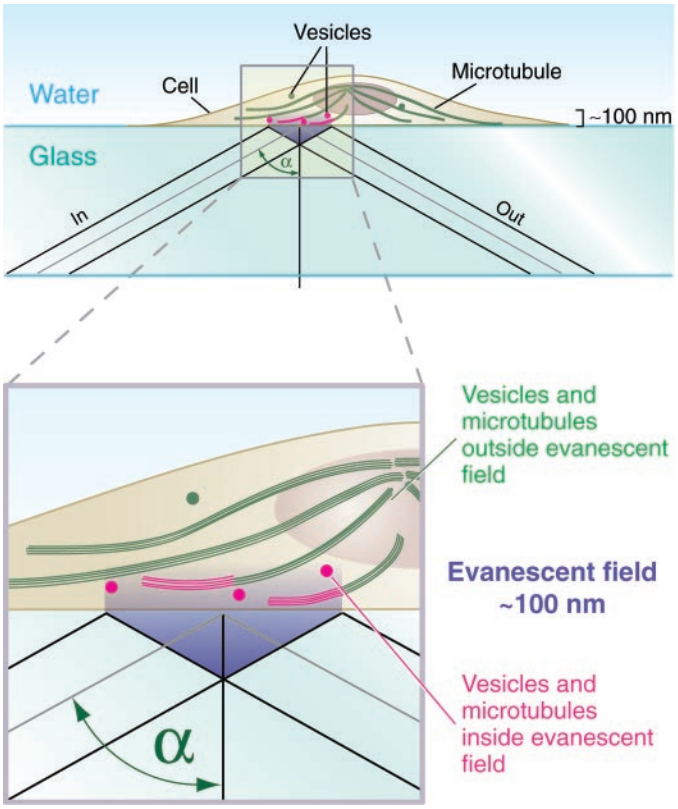

Fig. 4. An evanescent field occurs when incident light passes from a medium of high refractive index (glass) to one of low refractive index (water or a cell). Total internal reflection occurs when the angle of incident light exceeds a critical angle α. This field decays rapidly and, therefore, only illuminates ~100 nm of the sample closest to the coverslip. This enables specific visualization of only those fluorophores in direct proximity to the plasma membrane (shown in red), not those lying further away (green). This illumination mode can be coupled with conventional widefield microscopy to allow combined imaging of events close to and away from the coverslip.

Fluorescence resonance energy transfer (FRET). Intermolecular interactions form the basis of all processes in live cells and can be monitored by measuring the proximity of one component to another. In the context of light microscopy, this can be achieved with the use of FRET. FRET occurs when two spectrally overlapping fluorophores are very close together and in an orientation such that dipole-dipole coupling results in a transfer of energy from one probe to another (*47*). Because the efficiency of FRET depends on the inverse sixth power of the distance between the donor and acceptor (*47*), this allows measurement of protein-protein interactions in live cells. Limitations of this approach are that FRET is extremely inefficient, and many hypothetical FRET pairs do not produce FRET in live cells. The most reliable and reproducible examples of FRET occur when the donor and acceptor fluorophores are covalently linked to one another (*48*). Excellent examples of this include the elimination of FRET after caspase cleavage of a linker between donor and acceptor molecules (*49*) and the application of FRET to biosensors measuring intracellular processes such as calcium flux (*7*). Further developments of FRET pairs and improvements in imaging methods (*50*) will doubtless enhance the applicability of FRET to live cell studies.

Fluorescence lifetime imaging (FLIM). The detection of fluorescent probes is typically achieved by counting the number of photons emitted by the excited state of a fluorophore. An alternative approach is to measure the lifetime of this excited state with the use of FLIM (*51*). This provides a means for detecting multiple fluorophores in live

cells including spectrally related molecules such as GFP variants (*52*), which have different fluorescence lifetimes despite substantially overlapping spectra. FLIM provides an excellent means for measuring FRET because the lifetime of the excited state decreases greatly when FRET is occurring (essentially there is an additional means for decay from the excited state). FLIM measurement of FRET has recently been applied to the imaging of kinase activation (*53*). However, there are a number of limitations of the approach: resolution is limited, and FLIM is extremely difficult to perform on live cells. Despite the advent of commercial add-on packages for confocal microscopes, a key limitation remains that FLIM is technically very demanding and also requires complicated mathematical analysis of results.

Conclusions and Perspectives

The rapid development of live cell microscopy has required input from biologists, who have provided new fluorescent probes that can be easily adapted to study myriad different proteins, and physicists, who have driven the improvement in microscope systems and software. These contributions have led to unprecedented access to sophisticated imaging technology. For example, many researchers who have no previous microscopy experience may now use a departmental confocal microscope. It is clear that research in specialist laboratories will continue to drive developments in light microscopy. The resolution attainable by light microscopy is being enhanced by recent developments in imaging that break the diffraction limit (*54*), and such approaches can be applied to live cells. Recent work using stimulated emission depletion (STED) to quench excited fluorophores at the rim of the focal illumination spot has enabled a substantial increase in resolution to below the diffraction limit, giving a spot size of 100 nm (*55*). 4Pi confocal microscopy, in which two opposing objective lenses are used to sharpen the point spread function of illumination (*56*), has been used for live cell imaging, and incorporation of STED with 4Pi microscopy has reduced the spot size to 33 nm (*57*). Computational adaptive optics, widely used by astronomers, can be used to correct for changes in refractive index within thick specimens (*58*). Alternative approaches to increase attainable resolution include scanning near-field optical microscopy (SNOM, also known as NSOM). SNOM is similar to atomic force microscopy in that a sharp probe physically scans the surface of the sample; it can also be coupled to fluorescence imaging (*59*), where excitation light is guided through this probe, and its application to living cells is under development.

The field of live cell imaging is also of great interest to pharmaceutical and biotechnology companies (*60*). Many are now developing high-throughput and high-content screening platforms for automated analysis of intracellular localization and dynamics. This is paralleled with the increasing development of fluorescent biosensor assays that provide an optical readout of a physiological effect, often based on GFP technology or bioluminescence. Clearly, future developments in this field will be of great interest and benefit to both biotechnology and curiosity-driven research.

References and Notes

1. C. L. Rieder, A. Khodjakov, *Science* **300**, 91 (2003).
2. D. W. Piston, S. M. Knobel, *Trends Endocrinol. Metab.* **10**, 413 (1999).
3. J. Lippincott-Schwartz, G. H. Patterson, *Science* **300**, 87 (2003).
4. A. F. Fradkov *et al.*, *FEBS Lett.* **479**, 127 (2000).
5. C. Janetopoulos, T. Jin, P. Devreotes, *Science* **291**, 2408 (2001).
6. T. Balla, P. Varnai, *Sci. STKE* **2002**, pl13 (2002), http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2002/125/pl3.
7. A. Miyawaki *et al.*, *Nature* **388**, 882 (1997).
8. J. C. Simpson, V. E. Neubrand, S. Wiemann, R. Pepperkok, *Histochem. Cell Biol.* **115**, 23 (2001).
9. B. A. Griffin, S. R. Adams, R. Y. Tsien, *Science* **281**, 269 (1998).
10. G. Gaietta *et al.*, *Science* **296**, 503 (2002).
11. K. Stroffekova, C. Proenza, K. G. Beam, *Pflug. Arch.* **442**, 859 (2001).
12. R. P. Haugland, *Handbook of Fluorescent Probes and Research Products* (Molecular Probes, Eugene, OR, ed. 9, 2002).
13. C. M. Waterman-Storer, A. Desai, J. C. Bulinski, E. D. Salmon, *Curr. Biol.* **8**, 1227 (1998).
14. G. H. Patterson, J. Lippincott-Schwartz, *Science* **297**, 1873 (2002).
15. T. J. Mitchison, *J. Cell Biol.* **109**, 637 (1989).
16. R. Ando, H. Hama, M. Yamamoto-Hino, H. Mizuno, A. Miyawaki, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 12651 (2002).
17. J. B. Pawley, in *The Handbook of Biological Confocal Microscopy* (Plenum, New York, ed. 2, 1995).
18. W. B. Amos, in *Protein Localization by Fluorescence Microscopy: A practical approach*, V. J. Allan, Ed. (Oxford Univ. Press, Oxford, 2000), pp. 67–108.
19. Technical Note 14 (Roper Scientific, Trenton, NJ, 2002), available at www.roperscientific.com/pdfs/technotes/onchipgain.pdf.
20. M. Straub, P. Lodemann, P. Holroyd, R. Jahn, S. W. Hell, *Eur. J. Cell Biol.* **79**, 726 (2000).
21. W. Wallace, L. H. Schaefer, J. R. Swedlow, *Biotechniques* **31**, 1076 (2001).
22. J. R. Swedlow, K. Hu, P. D. Andrews, D. S. Roos, J. M. Murray, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 2014 (2002).
23. J. R. Swedlow, I. Goldberg, E. Brauner, P. K. Sorger, *Science* **300**, 100 (2003).
24. J. A. Steyer, W. Almers, *Biophys. J.* **76**, 2262 (1999).
25. J. B. Pawley, *Scanning* **24**, 241 (2002).
26. W. Denk, J. H. Strickler, W. W. Webb, *Science* **248**, 73 (1990).
27. F. Helmchen, M. S. Fee, D. W. Tank, W. Denk, *Neuron* **31**, 903 (2001).
28. R. K. Jain, L. L. Munn, D. Fukumura, *Nature Rev. Cancer* **2**, 266 (2002).
29. R. H. Christie *et al.*, *J. Neurosci.* **21**, 858 (2001).
30. P. Ray *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 3105 (2002).
31. J. C. Wu, M. Inubushi, G. Sundaresan, H. R. Schelbert, S. S. Gambhir, *Circulation* **105**, 1631 (2002).
32. T. F. Watson, M. A. Neil, R. Juskaitis, R. J. Cook, T. Wilson, *J. Microsc.* **207**, 37 (2002).
33. D. G. Weiss, W. Maile, R. A. Wick, W. Steffen, in *Light Microscopy in Biology: A practical approach*, A. J. Lacey, Ed. (Oxford Univ. Press, Oxford, 1999), pp. 73–150.
34. J. S. Ploem, F. A. Prins, I. Cornelese-ten Velded, in *Light Microscopy in Biology: A practical approach*, A. J. Lacey, Ed. (Oxford Univ. Press, Oxford, 1999), pp. 275–310.
35. D. Zicha, E. Genot, G. A. Dunn, I. M. Kramer, *J. Cell Sci.* **112**, 447 (1999).
36. J. A. Steyer, W. Almers, *Nature Rev. Mol. Cell Biol.* **2**, 268 (2001).
37. C. J. Merrifield, M. E. Feldman, L. Wan, W. Almers, *Nature Cell Biol.* **4**, 691 (2002).
38. S. E. Sund, D. Axelrod, *Biophys. J.* **79**, 1655 (2000).
39. A. Ishijima, T. Yanagida, *Trends Biochem. Sci.* **26**, 438 (2001).
40. Y. Sako, S. Minoghchi, T. Yanagida, *Nature Cell Biol.* **2**, 168 (2000).
41. G. Seisenberger *et al.*, *Science* **294**, 1929 (2001).
42. P. Schwille, U. Haupts, S. Maiti, W. W. Webb, *Biophys. J.* **77**, 2251 (1999).
43. J. Lippincott-Schwartz, E. Snapp, A. Kenworthy, *Nature Rev. Mol. Cell Biol.* **2**, 444 (2001).
44. T. Misteli, *Science* **291**, 843 (2001).
45. R. D. Phair, T. Misteli, *Nature Rev. Mol. Cell Biol.* **2**, 898 (2001).
46. K. Hirschberg *et al.*, *J. Cell Biol.* **143**, 1485 (1998).
47. L. Stryer, *Annu. Rev. Biochem.* **47**, 819 (1978).
48. R. Y. Tsien, A. Miyawaki, *Science* **280**, 1954 (1998).
49. X. Xu *et al.*, *Nucleic Acids Res.* **26**, 2034 (1998).
50. T. Zimmermann, J. Rietdorf, A. Girod, V. Georget, R. Pepperkok, *FEBS Lett.* **531**, 245 (2002).
51. J. R. Lakowicz, H. Szmacinski, K. Nowaczyk, K. W. Berndt, M. Johnson, *Anal. Biochem.* **202**, 316 (1992).
52. R. Pepperkok, A. Squire, S. Geley, P. I. Bastiaens, *Curr. Biol.* **9**, 269 (1999).
53. T. Ng *et al.*, *Science* **283**, 2085 (1999).
54. E. H. Stelzer, *Nature* **417**, 806 (2002).
55. T. A. Klar, S. Jakobs, M. Dyba, A. Egner, S. W. Hell, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 8206 (2000).
56. H. Kano, S. Jakobs, M. Nagorni, S. W. Hell, *Ultramicroscopy* **90**, 207 (2001).
57. M. Dyba, S. W. Hell, *Phys. Rev. Lett.* **88**, 163901 (2002).
58. M. J. Booth, M. A. Neil, R. Juskaitis, T. Wilson, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 5788 (2002).
59. F. de Lange *et al.*, *J. Cell Sci.* **114**, 4153 (2001).
60. R. A. Blake, *Curr. Opin. Pharmacol.* **1**, 533 (2001).
61. Materials and methods are available as supporting material on *Science* Online.
62. D.J.S. is supported by a Research Career Development Award from the Medical Research Council (grant G120/617). Figure 2 was produced with the use of equipment provided by the Biotechnology and Biological Sciences Research Council, UK Bioimaging Initiative (grant C11195) and the Wellcome Trust (grant 043846) to V.J.A.

Supporting Online Material

www.sciencemag.org/cgi/content/full/300/5616/82/DC1
Materials and Methods
Movies S1 and S2