

Fluorescence microscopy today

Rafael Yuste

Fluorescence microscopy has undergone a renaissance in the last decade. The introduction of green fluorescent protein (GFP) and two-photon microscopy has allowed systematic imaging studies of protein localization in living cells and of the structure and function of living tissues. The impact of these and other new imaging methods in biophysics, neuroscience, and developmental and cell biology has been remarkable. Further advances in fluorophore design, molecular biological tools and nonlinear and hyper-resolution microscopies are poised to profoundly transform many fields of biological research.

“Pendant le cours des trente dernières années, l’histologie a réalisé des progrès considérables. Ce serait presque justice de dire qu’elle a été renouvelée de fond en comble...” (*In the last thirty years, histology has achieved considerable progress. It would be almost fair to say that it has been renewed from top to bottom.*)

—P. Latteux, *Manuel de Technique Microscopique* (eds. Cocoz, A., Delahaye, A. & Lecrosnier, E.) (Paris, 1887).

Like most primates, humans are essentially a visual species. Our retinas capture most of the sensory information we gather from the world, and correspondingly, most of our sensory neocortex is engaged in the processing of visual inputs. Not surprisingly, visualization techniques are at the heart of the fields of science and engineering. In the biological sciences in particular, microscopes have been one of the most fundamental tools in our laboratories since Leeuwenhoek, and the examination of microscopic specimens has been a cornerstone, not only of biological research, but also of clinical pathology and medical diagnosis.

Unfortunately, living matter is not very suitable for direct optical examination

owing to its scattering properties in visible wavelengths and the light-protective mechanisms that exist in most species to prevent photodamage of internal tissues by the sun. For decades, microscopy specimens have most often been chemically fixed because of the stability of fixed tissue and improved optical transparency of biological tissues that have been dehydrated and refraction index-matched. Therefore microscopists, and biologists in general, have essentially attempted to guess how living tissues function based on the examination of fixed specimens. Like reconstructing a football game from a series of still pictures, this is an arduous (and some would say hopeless) endeavor¹.

This impasse has been recently superseded by the introduction of novel imaging techniques. For the purpose of our discussion, ‘imaging’ can be defined as the use of visualization techniques on living samples. Imaging is the new histology, the histology of living tissue, and it has developed from the fertilization of traditional microscopy by several new techniques that allow the systematic examination of living tissue. Life is dynamic by definition, and this dynamism, which attracts generation after generation of researchers to the life sciences, is an essential aspect that can be ideally monitored over time with imaging techniques.

Fluorescence microscopy has become an ideal microscopy technique for the examination of all biological specimens, fixed or alive, because it allows the selective and specific detection of molecules at

small concentrations with good signal-to-background ratio. At the same time, although traditional fluorescence microscopy affords excellent detection of fluorophores in thin samples, when applied to thick or living samples, it has always been hampered by the fact that the entire sample is excited indiscriminately and therefore most of the fluorescent photons arise from out-of-focus fluorophores. Confocal scanning microscopy, developed in the last several decades^{2,3}, solved this problem by restricting photodetection to light originating from the focal point. Thus, optical sectioning became possible and afforded three-dimensional microscopic reconstructions of biological specimens. At the same time, the rejection of out-of-focus light creates a situation whereby only a very small proportion of all emitted photons are actually detected. To increase the signal, higher excitation is needed, leading to photodamage and photobleaching. This Achilles’ heel of confocal microscopy has been partly alleviated by the development of spinning-disk scanners, based on the disk invented in 1884 by Nipkow⁴. In these instruments, the parallel use of many pinholes enhances the detection of the fluorescence and consequently reduces the amount of excitation needed and therefore, reduces the photodamage and photobleaching.

In the last decade, two techniques have revolutionized fluorescence microscopy: the development of genetically encoded fluorophores, such as GFP^{5,6} and the inven-

Rafael Yuste is at the Howard Hughes Medical Institute, Department of Biological Sciences, Columbia University, New York, New York 10027, USA.
e-mail: rmy5@columbia.edu

tion of two-photon microscopy⁷. GFP is a naturally fluorescent protein, originally cloned from the jellyfish, *Aequoria victoria*. The GFP gene can be expressed in any species in which genetic engineering is possible and can be used to fluorescently label proteins, and follow the movement of these fusion proteins. GFP has generated an enormous interest in biological research and in the industry⁸. Many variants of GFP have been synthesized or isolated from nature, providing a wide choice for microscopists⁹.

In addition, the last decade has seen the application of two-photon excitation to biological microscopy. Two-photon excitation occurs when two low-energy photons (normally infrared wavelengths) together excite a chromophore and generate fluorescence. Originally proposed as a *Gedanken* (thought) experiment in physics¹⁰, the development of ultrafast lasers allowed the design of two-photon microscopes⁷. Rather than a quantum curiosity, two-photon excitation has profound consequences for microscopy of living specimens. First, the light scattering cross-section of living tissue is less in the infrared, so the examination of fluorophores deep in living samples becomes possible, for the first time in history^{11,12}. Second, for two-photon absorption to occur, the local concentration of photons needs to be very high—something that essentially only occurs at the focal point of the microscope. Thus, two-photon excitation has ‘built-in’ optical sectioning and, in contrast to confocal microscopy, this is achieved without any out-of-focus excitation. Because of this, two-photon excitation allows the microscopist to selectively excite (or photochemically activate or inactivate) the sample only at the scan plane, avoiding the photodamage and photobleaching that would result from a comparable excitation with confocal microscopy. It is truly an ‘optical magic wand’—although it has the disadvantage of a worse spatial resolution than confocal microscopes because of the longer excitation wavelength—as spatial resolution is proportional to the wavelength. As a laser-scanning microscopy, two-photon is also generally a slow, serial imaging method (see below). Overall, two-photon microscopy is really uniquely suited for local photochemistry or *in vivo* imaging at depth and really has no advantage for transparent or very thin imaging applications, for which confocal or more traditional fluorescence microscopies are superior.

Besides GFP and two-photon, other fields of fluorescence microscopy have been greatly advanced in the last decade. For example, total internal reflectance fluorescence (TIRF), a form of excitation that takes advantage of the extremely short range of an evanescent optical wave, has permitted researchers to monitor the movement of single molecules in very thin samples (as an example of its use, see¹³). Also, progress in imaging deconvolution and analysis have allowed researchers to localize the position in the chromosomes of individual genes, something that has had a profound impact in cytogenetics¹⁴. Finally, the design and commercialization of microscopes that can systematically separate and individually track dozens of fluorophores has provided a multicolor experimental palette to cell biologists and biochemists.

There is reason to believe that GFP and two-photon microscopy are just the opening chords of a major transformation that microscopy will experience in the coming years and decades. In the short- or medium-term future, there are many other technical developments that are poised to have an impact similar to these two techniques. Besides continued improvements in the design of lasers and light sources, further imaginative developments of genetically encoded fluorophores are pushing the applicability of fluorescence microscopy to novel grounds. For example, newer GFP designs are making possible the optical study of protein-protein interactions (split GFPs)¹⁵ or monitoring second-messenger cascades in living cells¹⁶. Also, split-GFP approaches allow the generation of an optical Boolean logic to monitor promoter networks¹⁷. GFP-based indicators of intracellular calcium, second messengers, pH and voltage are becoming very useful (for example, see¹⁸). In addition to GFP, new GFP-like proteins with potentially wide application have been isolated from coral¹⁹. Also, there are now several competing approaches to label proteins with (partly or fully) genetically encoded fluorophores or peptide tags^{20–23}. Even lipids can now be endogenously tagged with fluorophores²⁴.

Another exciting fluorophore development is the synthesis of fluorescent nanocrystals, or quantum dots²⁵ (‘Qdots’). These are inorganic fluorophores, whose semiconductor properties endow them with relatively broad excitation but narrow emission profiles. Furthermore, quantum dots can be made photochemically inert

and extremely resistant to photobleaching. Thus, they have high photostability and a narrow emission band tunable simply by particle size—like a quantum particle-in-a-box. Therefore, one can distinguish several different labels by spectral imaging. In principle, nanocrystals can be designed for any arbitrary excitation or emission wavelength and can be coupled to proteins, so it is conceivable that future microscopes could simultaneously detect specific fluorescence from dozens of different classes of fluorophores. Qdots have already been applied to the detection of single molecules in living samples²⁶ and appear ideal for long-term *in vivo* imaging or for the reconstruction of the spatiotemporal dynamics of biochemical networks inside living cells.

On the two-photon front, there are also constant developments that can extend the usefulness of laser microscopy. One shortcoming of most two-photon microscopes is that the serial scanning of the sample is slow, making this technique slower than using spinning-disk confocals, for example. Some approaches to enhance temporal resolution of laser scanning microscopes include optical methods of multiplexing the excitation beam, the design of spinning disks for two-photon excitation²⁷ or the use of fast acousto-optic scanning methods. Also, new phase-shaping methods (scanning-less microscopy²⁸) can enable the simultaneous illumination of the sample with femtosecond light pulses, something that could help push the temporal resolution of this serial scanning technique.

Although it is not a fluorescence technique, second harmonic generation (SHG) is another nonlinear optical phenomenon, well known in physics and chemistry, which could have a major impact in biology²⁹. In SHG, high-infrared light intensity drives the lowest-order nonlinear polarizability of molecules (or groups of molecules) in the specimen so that coherent light of exactly double frequency (or half the wavelength) is emitted. Because the process can occur away from resonance frequencies, there is no absorption of light, thus avoiding complications of photochemistry. This phenomenon is rare and requires, like two-photon excitation, a high concentration of photons at the focal point, something that also gives it optical sectioning. SHG is particularly interesting because it only occurs where chromophores are oriented in noncentrosymmetric arrays, such as chromophores adsorbed

to biological membranes or other chemical interfaces. Thus, SHG is perhaps the only optical technique that is truly sensitive to biological membranes, something which makes it ideal for detecting changes in membrane potential. As many important biological processes, such as electrophysiological communication, detection and transduction of external molecules and cell-cell interactions occur at plasma membranes, SHG is likely to become a very useful tool for biologists.

Finally, novel approaches are challenging even the most basic assumptions of light microscopy, such as the diffraction limit of spatial resolution, always viewed as immutable. Hyper-resolution microscopies^{30–32}, or stimulated-emission depletion strategies³³, have pushed the spatial resolution of light microscopy to tens of nanometers in special cases. Although these new methods have not yet been applied systematically to living samples, they allow microscopists to achieve milestones that were supposedly unachievable, such as optically monitoring the spatiotemporal dynamics of molecules well below the wavelength of light.

As a finishing thought, it is interesting to note that the first microscopes were actually used by Leeuwenhoek to study living samples³⁴. After a long detour, armed with the power of genetic engineering, chemical

synthesis and a deeper understanding of the physics of light, microscopists today are coming back to their true roots, to directly document the dynamism of life.

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