



## How Microscopy Produces a Sharper Image

by Dr. Stephen Rawlings and Jacky Byatt

### SUMMARY

By dramatically reducing background interference and enabling high resolution and three-dimensional imaging of structures at greater depths within living tissues, confocal laser scanning and two-photon scanning microscopy have proved to be indispensable tools for biological research.

Significant advances in scientific research often follow the introduction of novel technologies. It is abundantly clear that the commercial introduction of the confocal laser scanning microscope in the late 1980s led to major

breakthroughs in our understanding of cell and tissue biology. The device overcame a number of limitations common to standard light microscopy, allowing the noninvasive collection of very thin, high-resolution optical sections. These sections

could be used to digitally reconstruct the three-dimensional structure of the sample, providing information that was not obtainable by other methods.

Likewise, the appearance of two-photon scanning microscopy in the late 1990s has had a dramatic impact on the biological sciences, offering improved resolution, increased tissue penetration and the ability to visualize in vitro processes in tissue without damaging the sample.

In standard light microscopy, a relatively large volume of the sample is illuminated, and the light gathered by the objective lens comes not only from the point of focus, but also from below and

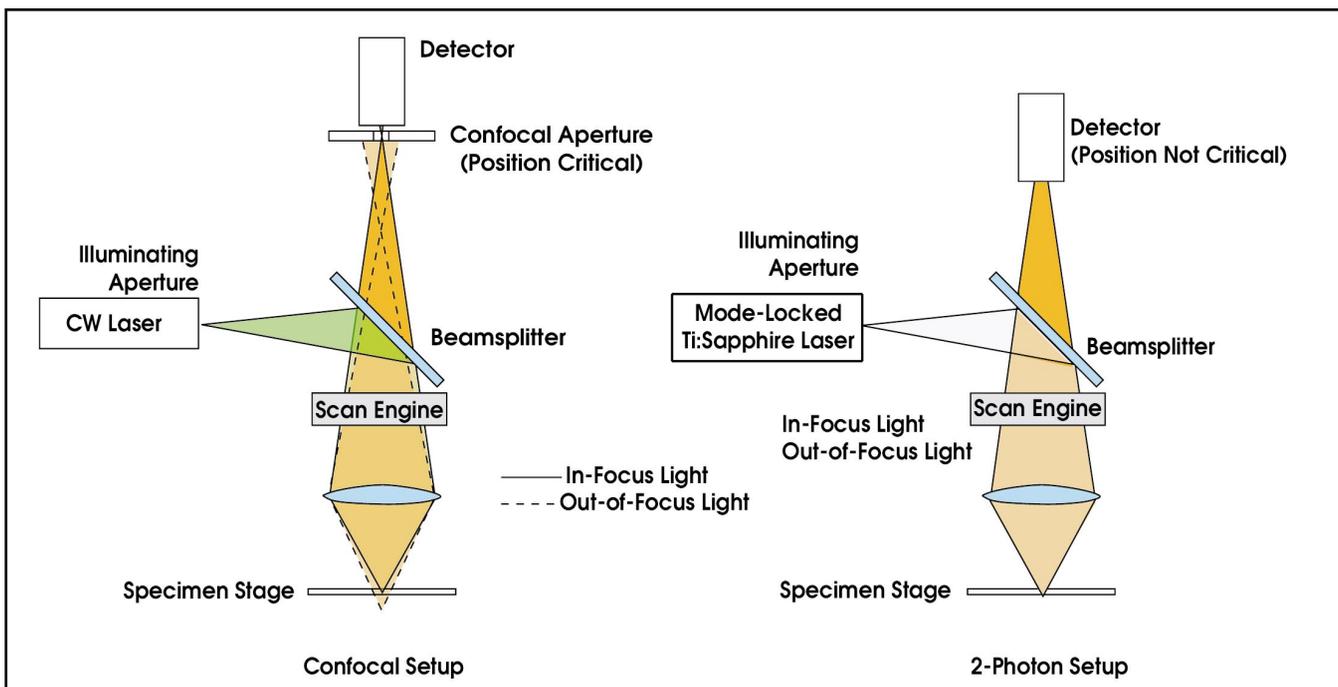


Figure 1. In a confocal microscope, the detector must be the same distance as the light source from the illuminated portion of the sample to effectively block out-of-focus light. The position of a detector in a two-photon system is variable.

above the focal plane. The resulting image contains the in-focus light as well as the haze or blur caused by the light from the out-of-focus planes. The basic principle of confocal microscopy is to eliminate the out-of-focus light, thus producing an accurate, sharp and high-resolution image. This is achieved by the use of pinhole apertures.

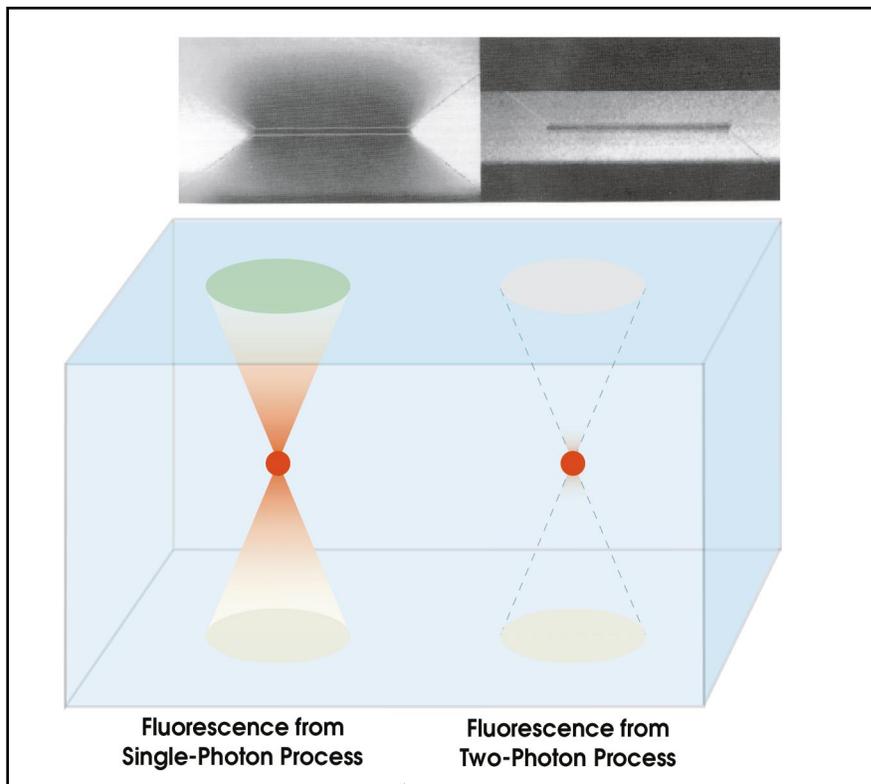
## A look inside

The first pinhole is placed in front of the light source to produce a distinct and spatially constrained illumination point. The light passing through this aperture is focused on the sample. The second pinhole is placed in front of the detector. If the optical distance from the detector aperture to the focal point is exactly the same as that between the focal point and the illuminating aperture (the confocal condition), only the light generated at the focal point will reach the detector; the pinhole will block the out-of-focus light.

The signal from the detector is then digitized and passed to a computer, which handles the data collection. Because the confocal microscope illuminates and collects only from a point source, the image has to be digitally built up by scanning the sample in the X and Y directions. The computer thus receives data from each illuminated point in the sample as it is scanned and uses software to reconstruct a digital image.

Although, in principle, a conventional light source can be used, continuous-wave (CW) visible sources — typically an argon or argon-krypton laser — are the norm. Lasers provide sufficient intensity to compensate for the light lost after passage through the detector pinhole and, by nature, provide a distinct and spatially constrained light point, eliminating the need for an illumination pinhole.

Two-photon microscopy setups are practically the same as confocal microscopy setups, except that the CW laser is replaced with a mode-locked Ti:sapphire laser operating in the near-infrared. The laser produces tens of kilowatts of peak power in a train of low-energy pulses — approximately 10 nJ per pulse. It is tuned to a wavelength about twice that of the intended absorption wavelength of the sample, which requires a nonlinear two-photon (or more) process to excite the



**Figure 2.** Two-photon excitation is more precise than single-photon methods, which tend to induce large amounts of fluorescence outside the plane of interest.

chromophores. Because the cross section for the two-photon process varies as the inverse fourth power of the distance from the focus, only at the focal point will there be enough energy to induce fluorescence. The need for pinholes is eliminated because there is virtually no fluorescence outside of the focal volume.

To generate a two-dimensional planar image, the focused light must be raster-scanned in the X- and Y-axes through the sample. Commercial confocal systems employ several scanning mechanisms. The most common approach is to use two galvanometric mirrors — one each for X and Y — coupled with a flat-field  $f$ -Theta scanning lens. In general, galvanometric systems are relatively slow, requiring a second or so to produce a full-frame image, but systems now available have increased the rate to as much as 30 fps by using resonant galvanometers.

Scanning speed is important, particularly for real-time imaging of living biological samples. The faster the scan rate, the more observable the phenomena that occur with a short time constant. One

popular technique used to speed up scanning is to combine a galvanometric mirror with an acousto-optical deflector and to use adjustable slits rather than pinholes. This provides up to 480 images per second but produces lower resolution than pinhole systems.

Another technique uses the modified Nipkow disc system (Figure 3). The laser beam passes through two spinning discs, the first containing about 20,000 microlenses and the second containing pinholes in the same pattern. Light returning from the sample passes back through the pinhole and into a CCD camera. Because this system uses a series of pinholes on discs that are spinning at 1800 rpm, many points in the sample are illuminated simultaneously, making it possible to observe the image in real time. This technique is not suitable, however, for two-photon microscopy.

Both of these laser scanning methods provide major advantages over conventional microscopy:

- *Improved resolution:* up to 1.4 times greater than with standard microscopy.

# SCANNING MICROSCOPY

- **More accurate quantitative data:** Because the light is captured from a distinct focal plane, out-of-focus light makes minimal contribution to the measurements.
- **Ability to accurately study subcellular structures such as the nucleus.**
- **3-D reconstruction:** By collecting optical sections at different planes of focus,

computer software can be used to digitally reconstruct 3-D representations of the sample.

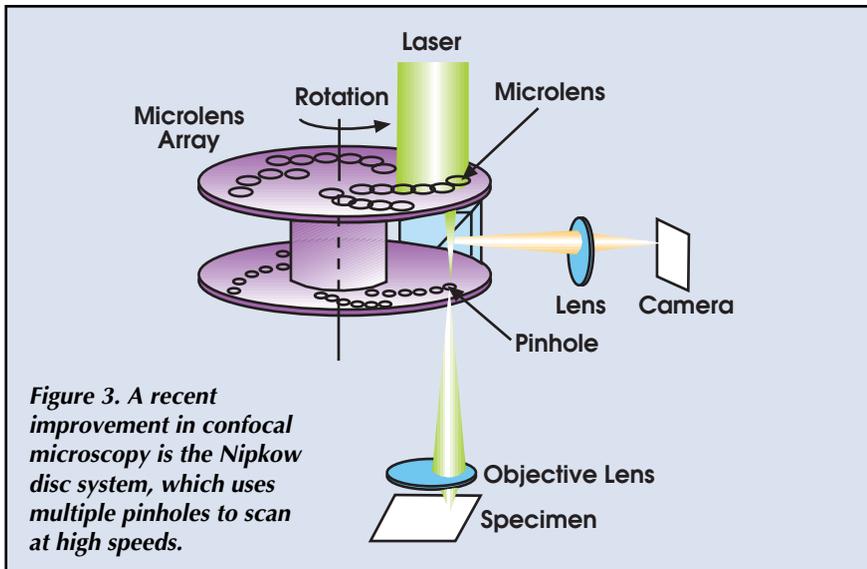
- **Noninvasive imaging:** The use of high-power laser illumination and the reduction in light-scattering artifacts allow non-invasive imaging of thick sections of semi-transparent tissues.

- **Collection of data in digital form:** The images are immediately available for processing and analysis by state-of-the-art computer software.

## And even better

Two-photon microscopy improves on the confocal microscope in several important ways:

- **Higher resolution:** All of the signal is generated at the focal point, not in the surrounding area.
- **Increased sensitivity:** Elimination of the pinhole allows all of the signal to reach the detector.
- **Increased tissue penetration:** Near-infrared light penetrates up to five times deeper into biological material than visible light.
- **Reduced photobleaching:** Chromophore fading is reduced at the focal point and eliminated in the surrounding areas.
- **Reduced toxicity:** Excited chromophores create toxic free radicals that can kill a living sample.
- **Dramatically reduced sample heating:** Although the peak amplitude of the mode-locked laser pulses is high, the average power is only a few tenths of a milliwatt,



**Figure 3.** A recent improvement in confocal microscopy is the Nipkow disc system, which uses multiple pinholes to scan at high speeds.

## Two-Photon Microscopy Has Permitted Observations of ...

### ... a growing intelligence

Two-photon laser scanning microscopy is helping Steve M. Potter and other researchers at the California Institute of Technology in Pasadena study the development of intelligence by coupling embryonic rat neurons to a virtual mouse in a virtual environment.

To do this, they grew dissociated neurons on a multielectrode array that can send and receive independent signals through 60 extracellular electrodes (see images on facing page). When grown in a culture, the dissociated neurons spontaneously connect and produce intricate forms of electrical activity. By exposing the neurons to stimuli, the researchers can induce changes in the pattern of connections and electrical activity. They are using this response to control a virtual mouse. For example, the neurons receive electrical feedback when the mouse approaches barriers. Using time-lapse two-photon microscopy, changes in the neural network can be recorded without damaging the neurons.

Potter is now at the Georgia Institute of Technology and Emory University.

### ... chemotherapeutics entering a cancer cell

At State University of New York in Buffalo, Xiaopeng Wang and his colleagues used multiphoton microscopy coupled with microspectrofluorimetry to study how chemothera-

peutic drugs and their carrier hormones enter into living cancer cells. By attaching a green-emitting fluorochrome to the drug (AN-152) and a red-emitting fluorochrome to its carrier hormone ([D-Lys<sup>6</sup>]LH-RH), they monitored the progress and distribution of both substances simultaneously as they penetrated the cell. The inherent localization ability of the microscopy technique enabled them to compare the effect of carrier vs. carrier/drug with regard to localization in different compartments of the cancer cells.

### ... NAD and NADH levels during translation

Multiphoton microscopy is helping a team led by Qinghong Zhang at Oregon Health Sciences University in Portland investigate the processes by which DNA coding is translated to individual cells. Nicotinamide adenine dinucleotide (NAD) and its hydride (NADH) are important intermediates in the biochemical oxidations and reductions that provide chemical energy within the cell; they can facilitate the protein binding process that determines which characteristics are passed on.

The researchers used two-photon microscopy to measure the concentrations of NAD and NADH, and to see what effects changes in the NAD/NADH ratio had on binding the corepressor CtBP to cellular and viral transcription repressors.

orders of magnitude less than that of the lasers used in confocal microscopy.

- *Handles UV:* Ultraviolet absorption bands may be addressed without the debilitating effects of a UV laser.

The main drawback is the cost of the mode-locked laser system, which is three or more times that of the typical laser used in confocal microscopy.

Although confocal microscopes are often used in reflected-light mode in industrial applications, they are rarely used this way in biological studies. In biological research, they are generally used in an epifluorescent mode. The sample is labeled with a fluorescent probe, laser light of a particular wavelength is used to excite the probe, and the detector records the resultant fluorescent light. By using specific probes, the distribution of a myriad of cellular components (organelles, proteins, nucleic acids) can be visualized and their subcellular or tissular distribution recorded.

With carefully selected fluorescent probes and lasers of different excitation wavelengths, it is possible to accurately study the colocalization of various cell components within the same sample, in-

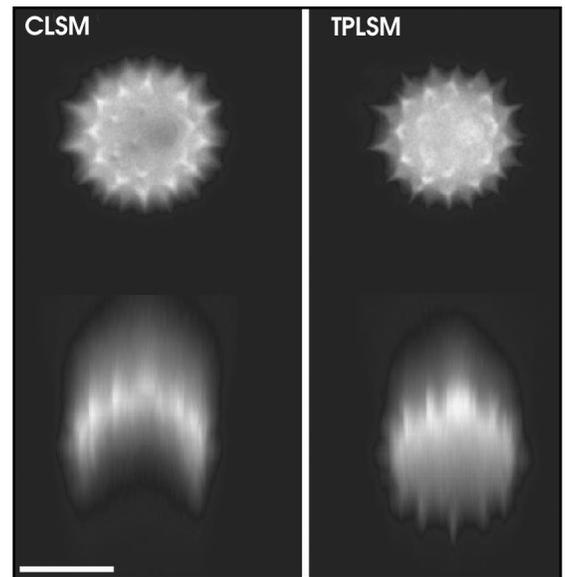
cluding the combination of fluorescent and transmitted light images. Rapidly scanning microscopes and ion-specific probes also enable accurate, real-time study of ion movement; e.g., calcium-ion fluxes, which play a major role in many cellular functions.

Laser scanning microscopy has generated a great deal of excitement in the research community. With the recent availability of more affordable confocal and two-photon microscopes, this technology has rapidly become the technique of choice for many researchers, particularly in the biosciences. □

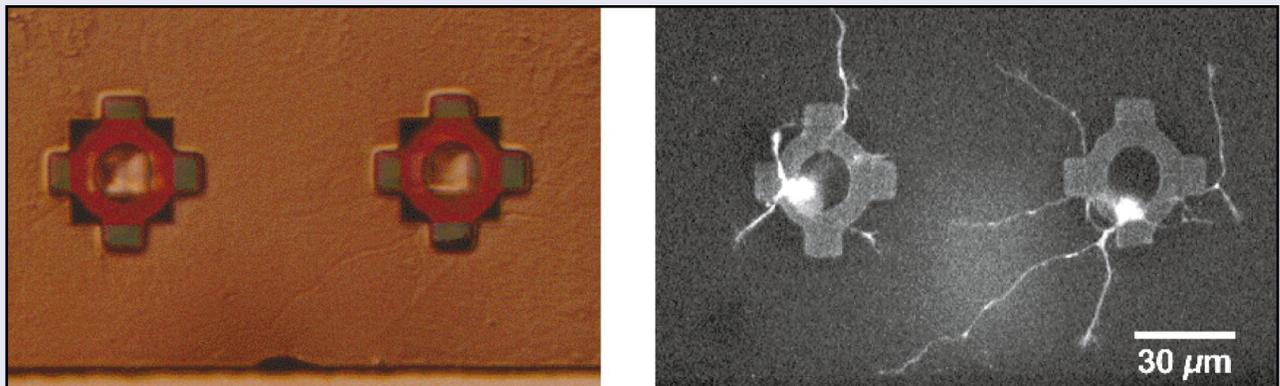
### Meet the authors

Stephen Rawlings is a freelance biological technologies consultant specializing in cell physiology.

Jacky Byatt is a business unit director for Melles Griot in Irvine, Calif.



**Figure 4.** In images of a pollen grain, a planar cross section (upper) shows that there are more out-of-focus spikes in the confocal microscope image than in that from the two-photon microscope. In a vertical cross section built from multiple planar images (lower), spikes are visible at the bottom of the two-photon microscope image, showing the deeper penetration of the near-infrared light. Courtesy of California Institute of Technology.



**Dissociated rat neurons one day after preparation on a multielectrode array image cannot be seen with a conventional Nomarski microscope (left). However, a projection through 10 1-µm layers using a two-photon microscope operating at 900 nm images the neurons without damaging them (right).**

### ... messenger RNA translated into protein

At the University of Pennsylvania in Philadelphia, researchers are studying how quickly proteins can be synthesized from messenger RNA in a dendrite and its subcellular structures. They transfected messenger RNA encoding green fluorescent protein (GFP) into the cell body and dendrites of living rat neurons, and used two-photon microscopy to measure the concentration of the newly synthesized GFP as a function of time. They discovered that GFP was observable

in the structures within 15 to 20 minutes after application of the messenger RNA (instead of several hours as expected) and that the rate of synthesis in isolated dendrites was exponential, whereas the rate in the cell bodies was linear. Also, the sites of GFP synthesis were immobile.

James Eberwine, the lead investigator, said that two-photon microscopy enabled them to monitor the translation continually without worrying about significant photobleaching. □