Lasers for **Microscopy**: Major Trends

In order for biologists, drug developers, clinical lab professionals and other scientists to fully exploit new microscopy techniques and applications, parallel developments in laser technology are often required. Laser manufacturers are responding with products optimized to match the needs of these applications.

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Laser development for the microscopy market continues to be driven by key trends in applications, which currently include superresolution techniques, multiphoton applications in optogenetics and other areas of neuroscience, and even a shift in multiphoton imaging toward preclinical and clinical usage.

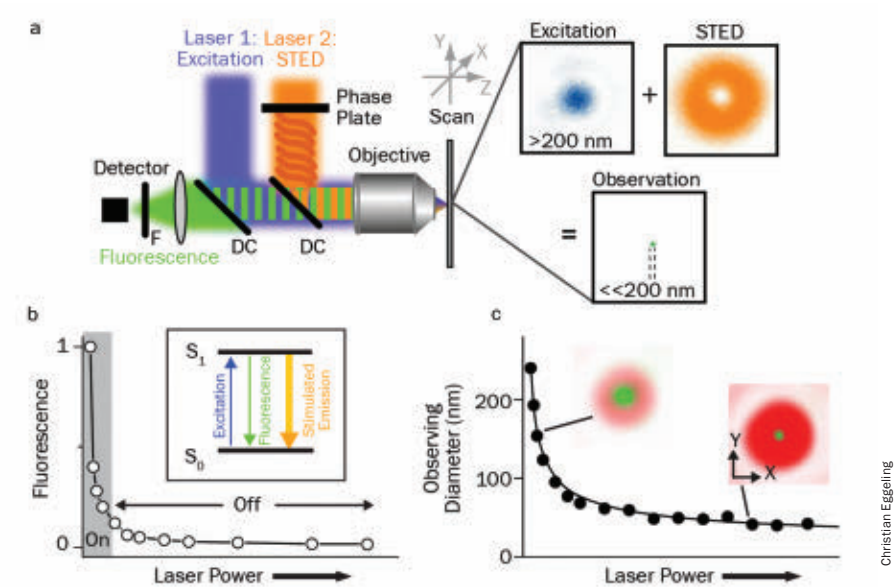
In spite of its long history, optical microscopy — particularly laser-based — is a very dynamic field. New techniques continue to be developed, while existing techniques are being applied to new applications.

Superresolution microscopy — optical switching of fluorescent labels

In order to better understand the details of processes like signaling and the control of gene expression, biologists need to correlate molecular-level events with macroscopic structures and dynamics. This has fueled rapid growth in superresolution microscopy techniques, often referred to as nanoscopy, that go beyond the classical spatial resolution limit set by diffraction. This limit is about half the wavelength of light (i.e., for visible light approximately 200 to 250 nm in the XY plane) and, in the case of confocal microscopy, about 500 nm in the Z direction. Most superresolution techniques use CW lasers, often with fast internal or external (on/off) modulation.

All optical superresolution or nanoscopy techniques are based on the principle of optically and reversibly preparing states of a fluorescence label that differ in their emission characteristics (e.g., a bright "on" and a dark "off" state). Based on their different mechanisms for this on/off photoswitching, optical nanoscopy methods can be loosely divided into two groups: those that directly improve microscope effective spatial resolution by deterministic photoswitching in space and time, and those that achieve the higher resolution by (random) stochastically switching single-molecule fluorescence on and off in space.

Examples of the first group include stimulated emission depletion (STED) microscopy (Figures 1 and 2), ground state depletion microscopy, reversible saturable optical fluorescence transition (RESOLFT) microscopy or parallelized RESOLFT microscopy, often denoted nonlinear, or saturated structured illumination microscopy (SIM). Examples of



Christian Eggeling

Figure 1. Principles of STED. Schematic drawing of the setup of a STED nanoscope with phase plate, objective lens dichroic mirror, fluorescence filter, detector, scanning device, and excitation and STED lasers with their focal intensity distribution **(right)**, and a representative, subdiffraction-sized observation area **(a)**. STED nanoscopy is based on inhibiting fluorescence emission by de-exciting the excited S_1 "on" state to the S_0 "off" ground state via stimulated emission. Increasing the power of the STED laser drives the inhibition into saturation **(b)**. Combined with an intensity distribution that features at least one intensity zero, this on/off fluorescence inhibition realizes subdiffraction-sized observation volumes — the volume in which fluorescence emission is still allowed **(green insets)** decreases with increasing STED laser power **(c)**.

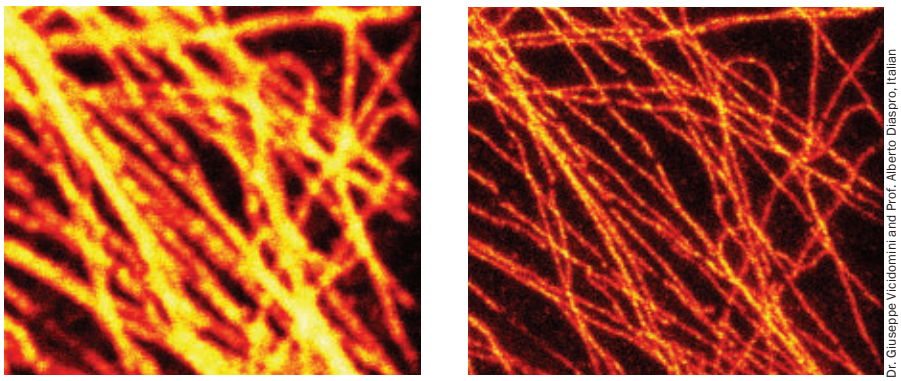
the latter include direct stochastic optical reconstruction microscopy (d-STORM) and fluorescence photoactivation localization microscopy (f-PALM).

Stochastic-based techniques such as f-PALM and d-STORM also use two lasers (Figure 3). Here, one laser is used to drive most of the fluorophores between the "on" and "off" states, so that only a very small, random subset of well-separated molecules are in their "on" state and available to be excited by a second laser. The fluorescence spread from each of these camera-imaged point sources is then analyzed to find the centroid (i.e., the point source or spatial localization, based on the point spread function, or PSF, of the microscope). Repetitive on/off switching brings up different random subsets in subsequent camera images for single-molecule localization. After many cycles, the microscope computer assembles all of the idealized point sources into a subdiffraction image, since the localization of a single molecule can be performed with high subdiffraction spatial precision.

Trends in CW lasers to support superresolution techniques

Laser manufacturers are supporting the rapid growth in superresolution microscopy with new products that provide higher and smoothly adjustable power, new wavelengths, and simplified fiber coupling and fiber combining.

Higher laser power is necessary because researchers want output in the 1- to 3-W range for STED, and typically a few hundred milliwatts for f-PALM and d-STORM. And in the case of SIM, microscope manufacturers are looking to move up to 300-mW lasers to decrease image acquisition times, whereas early users of this method worked with as little as 100 mW. Both deterministic and stochastic switching of fluorescence also need adjustable power output in the "bleaching" laser in order to optimize these techniques.



Dr. Giuseppe Vicidomini and Prof. Alberto Diaspro, Italian Institute of Technology

Figure 2. The effectiveness of STED is clear in this pair of images of fluorescently tagged microtubuli in mammalian cells using a 577-nm laser to drive the STED effect: conventional confocal **(left)** and gated STED image **(right)**.

Lasers based on optically pumped semiconductor laser (OPSL) technology are proving ideal to meet these higher/adjustable power needs, as OPSL technology is readily scalable simply by increasing the pump diode power. And the OPSL output can be smoothly adjusted from a few percent to maximum power with no effect on beam quality or beam pointing; this technology does not suffer from thermal lensing, which prevents wide power adjustment in diode-pumped solid-state (DPSS) and other solid state lasers.

Another key trend is the growing demand for new laser wavelengths, particularly longer ones, to optimally excite many of the new fluorophores. These include the m-Fruit series of fluorescent proteins and a host of new green, yellow and orange excited dyes originally developed for multichannel flow cytometry applications. And, as with wide-field microscopy, new nanoscopy methods often use combinations of multiple fluorophores to permit simultaneous mapping or probing for more than one type of structure or biochemical.

Wavelength scalability is yet another key advantage of OPSL technology. Lasers can be fabricated for any arbitrary output wavelength across much of the visible and near-UV spectrum merely by tweaking the design of the gain semi-

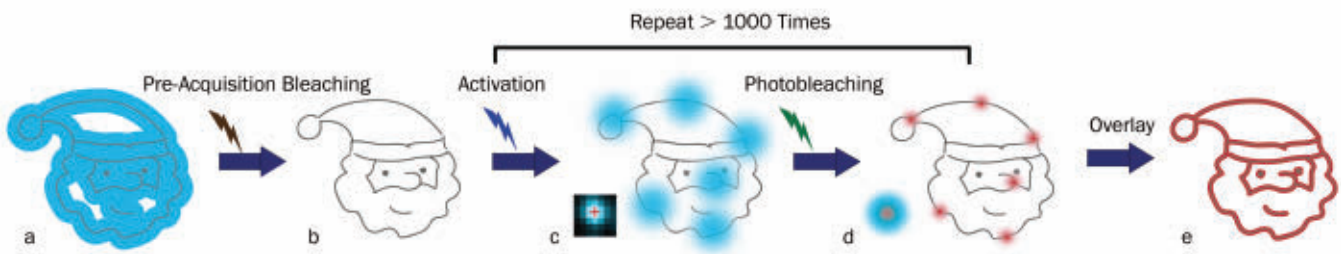


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Figure 3. Principles of STORM microscopy. A wide-field fluorescence image **(blue area)** is observed from overlapped fluorophores in a small target structure **(gray lines) (a)**. Little to no fluorescence is left after pre-acquisition bleaching with an appropriate laser **(b)**. Individual fluorophores are sparsely activated by an activation laser, and then emit fluorescence **(blue spots)** after applying an excitation laser (usually the same one for pre-acquisition bleaching) **(c)**. The excitation laser is again used to bleach the fluorophores. The center positions **(red crosses)** of the fluorophores are found using an appropriate molecule localization algorithm. The uncertainty of the center positions **(red spots)** is significantly smaller than the diameter of the single molecule fluorescence from the same fluorophores **(large blue spot in the left corner) (d)**. Repeating the activation, excitation, localization and photobleaching process more than 1000 times allows reconstruction (via overlaying the center positions of the fluorophores) of a superresolution image of the target structure **(red lines) (e)**.

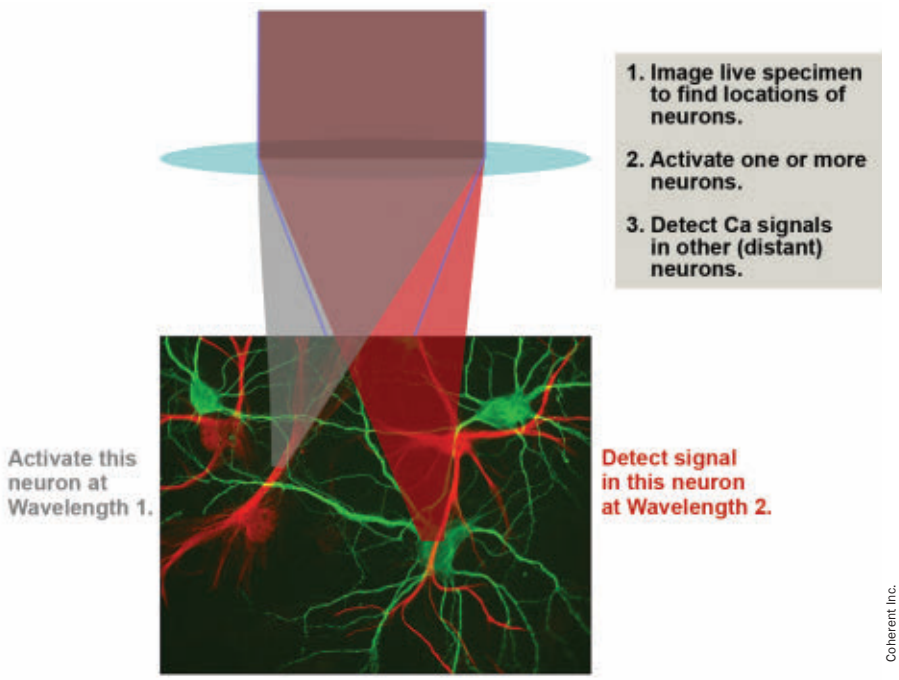

Figure 4. Advanced optogenetics. In all-optical physiology experiments with multiphoton lasers, neural activity can be manipulated and monitored at single neuron resolution. The use of two separate wavelengths for photoactivation and detection enables minimization of crosstalk between these 'write & read' channels.

conductor chip. For example, new lasers at 588 nm and 594 nm were recently introduced for exciting orange fluorophores. Other models at 550 nm have also been developed, as this wavelength can be used to simultaneously excite green dyes and several new yellow fluorophores with a single laser. Another new OPSL wavelength is the legacy (krypton ion) wavelength of 568 nm.

Longer (1050 to 1600 nm) laser wavelengths

In all-optical physiology experiments based on optogenetics, the outer cortex or even the neocortex in a live mouse brain is optically accessed by a laser microscope, usually via a small glass cover that replaces part of the skull. Specific neurons can be targeted and stimulated to fire using laser light. The activity of other neurons can then be observed and followed in real time via calcium probes. Moreover, by using femtosecond lasers to drive two-photon excitation for both the photostimulation and interrogation (Ca^{2+} imaging) parts of the research,

the entire experiment can be conducted in three dimensions at single-neuron resolution.

A key challenge in this type of experiment is to minimize crosstalk between the photostimulation and fluorescence excitation processes. One approach to this problem is to use photoactivators and fluorescent probes whose absorption peaks are well separated (Figure 4), i.e., with one <900 nm and one >1040 nm. Here, two ultrafast laser wavelengths are then focused into the sample. The first wavelength is used to activate neurons by two-photon excitation, and the second is used to image activity in other neurons, again via two-photon excitation.

In addition to requiring two independent laser wavelengths, this scheme requires the availability of photoactivators and calcium probes with well-separated spectra. For example, several red-shifted photoactivators have recently been added to the all-optical toolkit, including C1V1, ReaChR and Chrimson. The one-photon absorption peaks can be as long as 600 nm (Figure 5).

Ytterbium fiber femtosecond lasers for neuroscience

All-optical studies and more general red-shifted expressions or fluorophores have created a need for femtosecond lasers emitting multiple watts in the 1040- to 1150-nm range. These wavelengths are at or beyond the limit of the Titanium: Sapphire (Ti:S) lasers that have been the workhorses of multiphoton microscopy for the last 15 years. In addition, while a Ti:S-pumped optical parametric oscillator (OPO) can reach this wavelength range, the power is not sufficient for studying large neuron populations.

Ytterbium (Yb)-doped crystals are alternative gain materials, but power scaling causes problems due to cooling and thermal lensing of the bulk gain material. In response, laser manufacturers have developed a new type of femtosecond laser based on Yb-doped fiber that can be scaled to several watts, or even tens of watts, at 1030 to 1070 nm. While the first Yb-doped fiber lasers were unable to produce multiwatt powers with pulse durations shorter than ~300 fs, engineers at Coherent found proprietary solutions to address the trade-off between gain and nonlinearity in these high-power lasers. This has enabled the development of several series of lasers that provide the combination of high peak power and high average power and other advantages needed for all the experimental variations used for optogenetic photoactivation.

The most compact of these lasers are single-stage fiber oscillators that provide fixed wavelength output at the watt power level. These are ideal for photoactivation of the latest red-shifted opsins such as Chrimson or C1V1, while another shorter wavelength femtosecond laser (e.g., Ti:S-based) is then used for Ca^{2+} imaging in the 920- to 940-nm wavelength window, perhaps using a genetically encoded probe such as GCaMP. Or alternatively, the Ti:S laser can efficiently drive two-photon photoactivation at 940 nm via a short-wavelength opsin such as ChR2. The longer wavelength Yb-fiber laser can be used to image Ca^{2+} activity using a red-shifted genetic probe such as RCaMP. One example of this type of compact

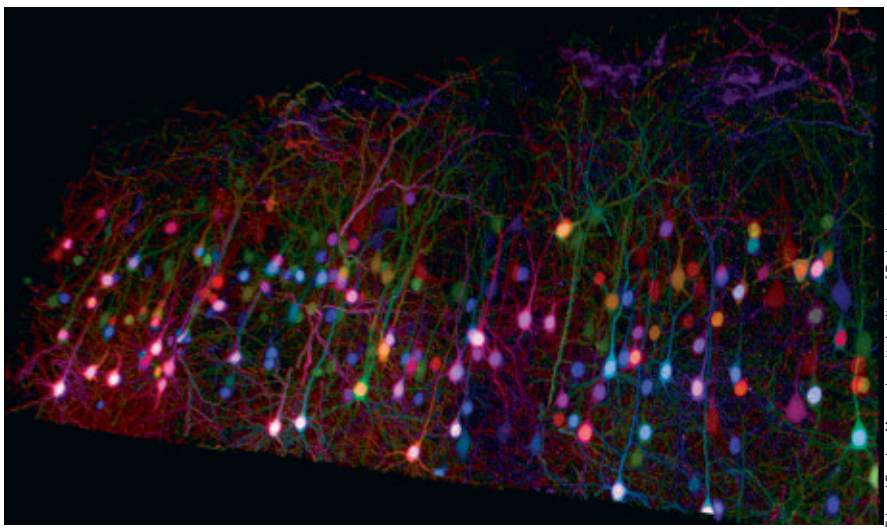

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Figure 5. The single-photon absorption spectrum of Chrimson is significantly red-shifted compared to other opsins.

fixed wavelength Yb fiber laser delivers over two watts of power at 1070 nm with a pulsewidth <55 fs.

Relating to tunability, some researchers need wavelength flexibility — for example, to finely optimize their experiments or to switch between using different opsins and calcium probes. By combining a Yb fiber laser with a tunable OPO in a single compact box, laser manufacturers now provide one-box sources that provide one to two watts of output, tunable across the entire near-IR. And in some of these, part of the fixed (1040 nm) wavelength Yb fiber power is directly available as an auxiliary output with the beam quality and short pulsewidth necessary to act as a second wavelength.

While two watts might be sufficient to activate 10 or possibly 20 neurons, experiments targeting tens or hundreds of neurons already could use higher power than this. Yb fiber laser architecture is power scalable in a classic master oscillator power amplifier (MOPA) configuration that has long been used to obtain high power in many other scientific and industrial laser types. Even just a single stage of amplification inside the laser can take the final power level up to a few tens of watts.

Laser manufacturers are also providing the combination of tunability and even high power by the combination of a Yb fiber laser and a tunable optical parametric amplifier (OPA). As Yb lasers are scaled into the tens of watts regime, pulse widths inevitably become a bit longer. However, a novel OPA architecture serves to provide tunability and eliminate this potential pulsewidth limitation.

Laser-based microscopy continues to be one of the most valuable tools in the life sciences. As researchers develop and refine new techniques and methods, laser sources need to be adapted and re-optimized in order to fully exploit the potential of these techniques. By closely working with key researchers and institutions, laser manufacturers are responding in a timely manner with new products designed specifically for the changing needs of these applications.

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