Modular Accessories Expand **Fluorescence Microscope Capabilities**

From neuroscience to morphogenesis and photosynthesis, optomechanical modules allow researchers to utilize fluorescence microscopy in diverse and increasingly complex studies.

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Despite the superior spatial resolution of complementary newer technologies such as atomic force microscopy (AFM), fluorescence microscopy remains a preeminent technology in the life sciences. Fluorescence detection offers a uniquely simple method of imaging with chemical specificity; for example, researchers can map the location and density of a specific protein or identify increased metabolic activity by imaging a chemical with a response that is modulated by local Ca^{2+} ion concentration. Moreover, the range of fluorescence labels has expanded beyond traditional dyes with an ever-growing arsenal of genetically encoded fluorescent probes and indicators. At the same time, the availability of new, economical, fiber-coupled lasers across the visible spectrum and beyond provides easy access to spatial, wavelength and temporal resolution far exceeding the capabilities of filtered lamps and LEDs (which still work well for wide-field illumination).

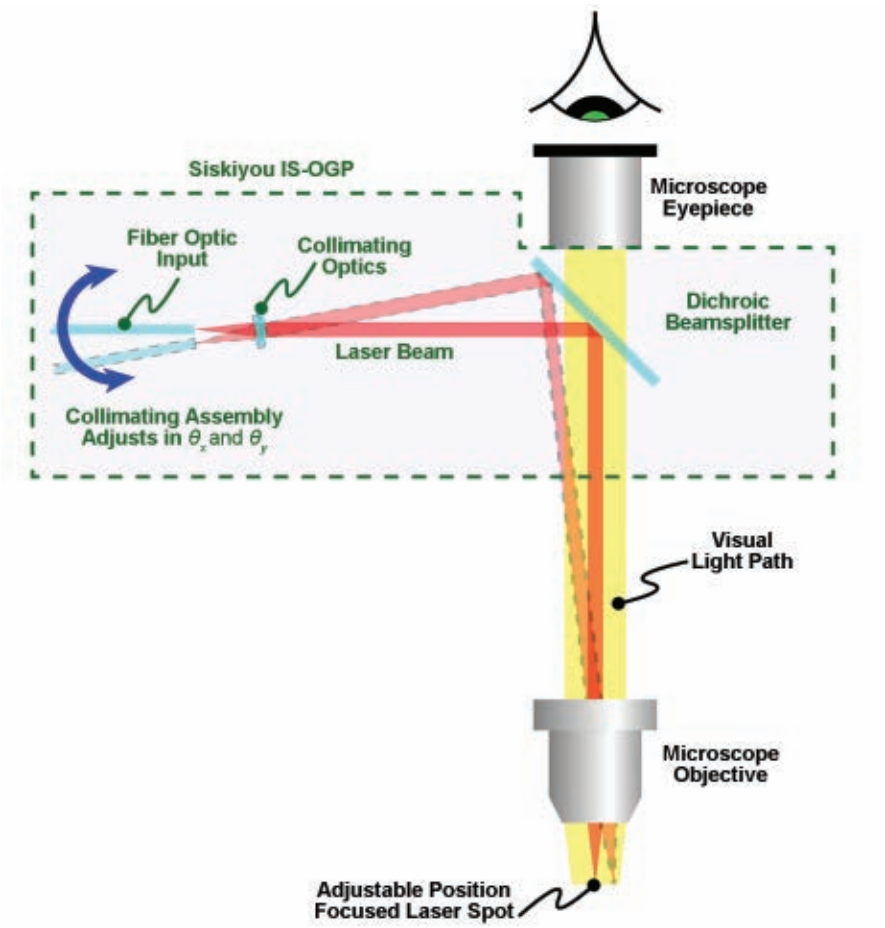

Figure 1. In fields such as optogenetics, researchers increasingly need to focus and maneuver a laser or other light source within the field of view of a fluorescence microscope. Courtesy of Siskiyou Corp.

Modular flexibility and adaptability

To support the latest experiments, there is a growing need for microscopes that offer functional flexibility and agility: the ability to add or interchange capabilities, without the high cost of a specialty microscope dedicated and optimized to a single imaging mode. For example, in optogenetics there is often a need to focus a laser into the sample. This need could be met with a confocal microscope, however some users will not need all of the expensive features that this device offers.

On the other hand, breadboard, or open cage, systems can provide such flexibility, but are often complex to align and lack the simplicity of a tube-based microscope based on a single optical axis. In response, manufacturers of precision optomechanical components and subsystems are developing a fast-growing array of interchangeable modules and accessories that allow users to customize the functionality of conventional microscopes — or even build their own. These plug-and-play modules can focus and independently maneuver one or more lasers or other light sources anywhere in the field of view, add filters to the excitation or imaging optics, add wide-field illumination sources, employ multiple cameras and associated spectral filters, and provide other optional functions.

Precision interchangeable beamsplitter module

Many applications need a means to intercept the optical path somewhere in the microscope's infinity space. For example,

a researcher may want to selectively sample part of the fluorescent image with an additional camera, based on wavelength and/or polarization. Or an experiment may call for the insertion of an additional filter, again based on polarization or wavelength into the primary beam path. Alternately, a user may want to add an additional wide-field light source in an epifluorescence configuration.

A new type of beamsplitter module based on a monolithic cube geometry can now intercept the light path without compromising optical and/or mechanical integrity. This type of module is machined from a single block of metal, providing maximum stability and alignment precision while also providing open access from four of the six faces of a theoretical cube. In its simplest implementation, this type of module enables a 45° beamsplitter to be inserted into the beam. This can be used to incorporate a second camera into the microscope, typically just below the trinocular assembly. However, users often need to insert, remove or exchange filters from experiment to experiment, or even in a single experiment. To this end, a self-registering slide accessory that directly mates to the beamsplitter module offers further flexibility. This allows a 45° beamsplitter or mirror to be smoothly inserted or removed from the microscope tube without disruption or misalignment of the optical path, permitting various filters to be used interchangeably in the excitation light and/or imaging path.

Optogenetics laser illumination module

There is a growing need to focus and maneuver a laser beam in the field of view of a fluorescence microscope. In optogenetics, this is necessary to selectively photoactivate target neurons or groups of neurons while studying their activity patterns using fluorescent imaging or patch clamp electrodes. A simple, turnkey module can now provide that capability (Figure 1). An example of this type of module is the IS-OGP optogenetics module from Siskiyou, which enables users to introduce a fiber-coupled laser beam into the infinity space of any leading optical microscope with a trinocular setup (including those from companies such as Nikon, Olympus and Zeiss). Moreover, the user can then position the focused laser stimulation spot (10 µm typical diameter with 20× objective) with submicron accuracy, anywhere within the field of view.

Figure 2. A different type of interchangeable objective assembly provides superior access and stability for applications such as those incorporating patch clamp measurements. Courtesy of Siskiyou Corp.

Standardization is key to wide applicability in any plug-and-play scenario. So this module accepts a single-mode, FC connectorized fiber optic cable input from the light source, thus enabling rapid connection and "hot-swapping" of any single-mode, fiber-coupled laser. The system then internally collimates the fiber output and introduces it into the microscope through a user-specified dichroic beamsplitter. Independent X and Y axis positional control of the focused laser spot is provided by two differential drive screws that permit scanning and positioning of a focused laser beam anywhere in the microscope's field of view. These can be operated manually or automatically using DC servo drivers.

Interchangeable Objective Assembly

Some of the modules for fluorescence microscopes provide a different approach to existing optional capabilities in order to provide specific practical benefits. An example of this is a type of interchangeable objective assembly designed for restricted working spaces and distances, or when the lateral motion of a traditional objective turret could disturb the sample. This is particularly useful when a fluorescence microscope is combined with patch clamp (electrophysiology) technology.

A typical example would be to use focused laser or LED illumination to photoactivate opsins in one or more neurons and then use patch clamp electrodes to directly measure the membrane voltage in an adjacent neuron. Not only does this optomechanical module (Figure 2) provide a low profile, the motion is about an axis that intersects the optical microscope's main axis, unlike a conventional turret, where the axis is necessarily offset.

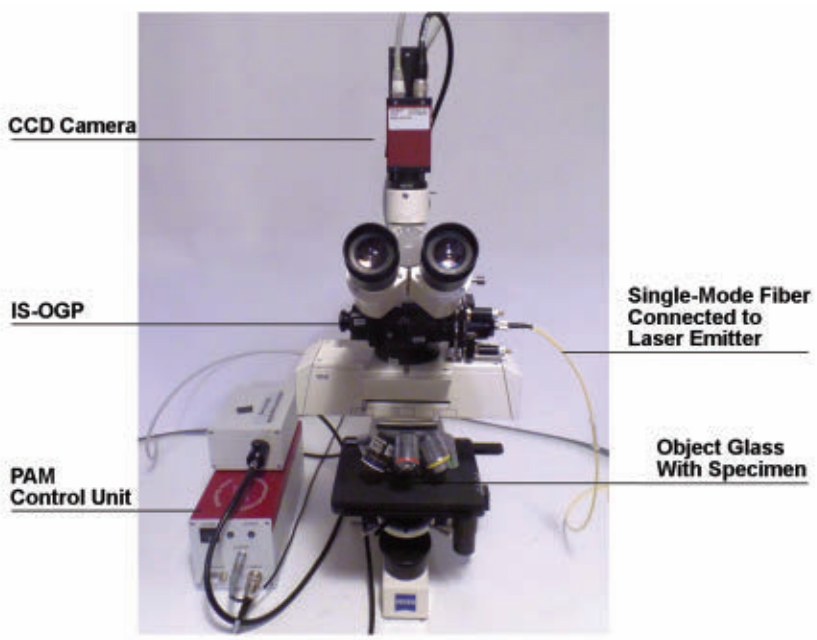

Figure 3. The microscope arrangement used by Johannes Goessling and co-researchers in their photosynthesis studies. The use of an IS-OGP laser excitation module enables a fiber-coupled laser to be focused and maneuvered across the field of view of a commercial microscope optimized for detecting chlorophyll fluorescence. Courtesy of Johannes Goessling.

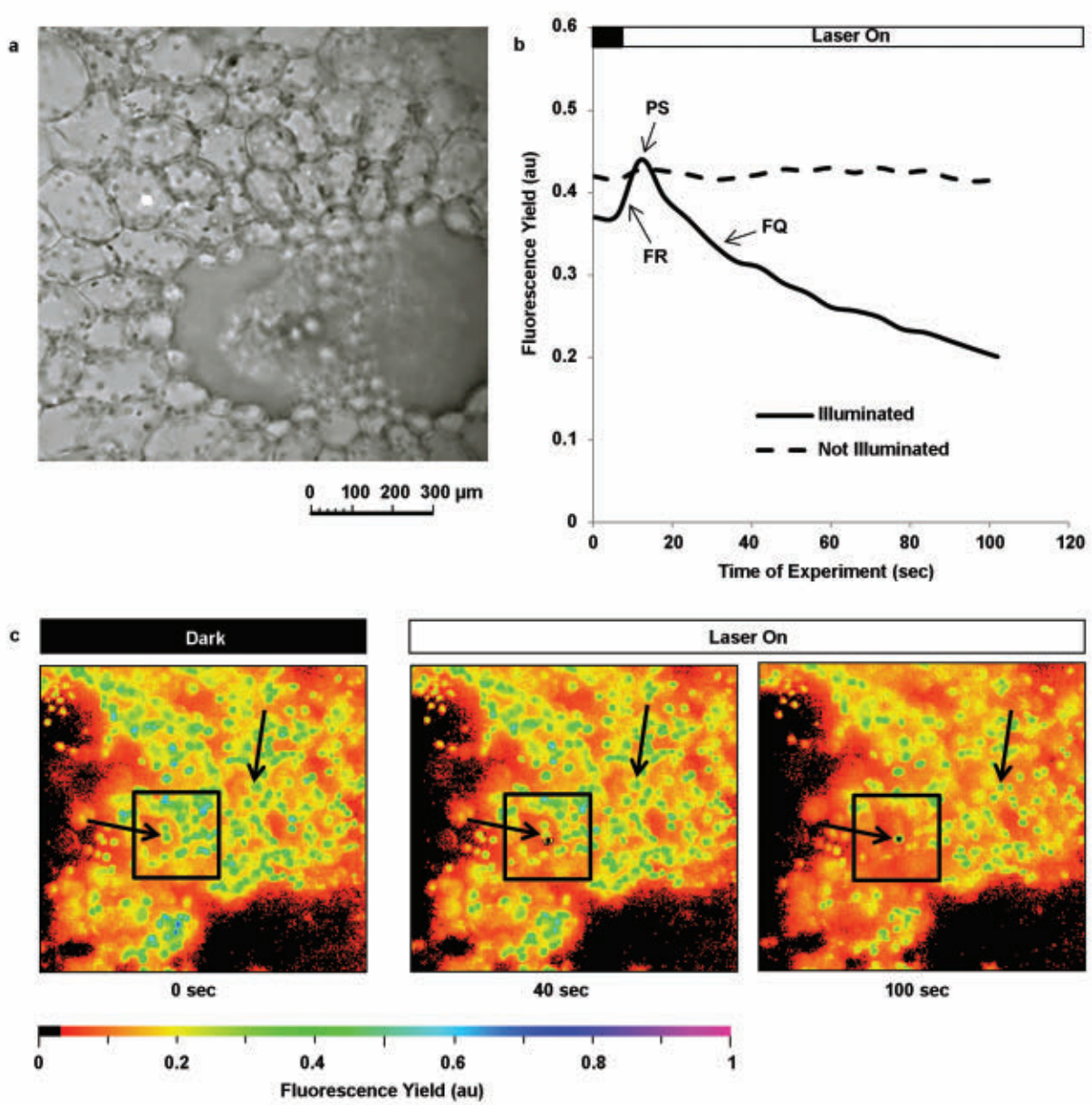

Figure 4. A focused spot of 650-nm laser light enables photosynthesis to be turned on and off with high spatial and temporal resolution. When the laser is first turned on, fluorescence increases in that location before photosynthesis, and then effectively quenches fluorescence. Courtesy of Johannes Goessling.

Laser excitation for studying single-cell photosynthesis

A recent set of studies of photosynthesis at the University of Copenhagen shows how other, very different areas of life sciences can benefit from adding laser beam steering functionality to a microscope. These studies have been conducted by Johannes Goessling, a graduate student in the research group of professor Michael Kühl.

In phototrophic organisms, light harvesting for photosynthesis primarily involves chlorophyll a — a pigment that is an efficient absorber of visible light with absorption maxima at blue and red wavelengths. Photosynthesis takes place in specialized organelles called chloroplasts, where most of the absorbed light is channeled toward the photosystems, while a small fraction of energy is re-emitted as chlorophyll fluorescence. Changes in chlorophyll fluorescence during illumination with actinic light can be used to estimate the photosynthetic activity.

In their work, the researchers inserted an IS-OGP laser excitation module between the filter cube slider and the eyepiece trinocular of an epifluorescence microscope (Figure 3). This microscope is already configured for studying single-cell photosynthesis from a specimen by means of a dedicated chlorophyll fluorescence imaging system (from Heinz Walz GmbH) that includes three (RGB) filtered excitation sources (LEDs) and a CCD camera. By pulse amplitude modulated non-actinic measuring of light, spatially resolved chlorophyll fluorescence can be monitored over the entire field of view with this type of setup. Switching the excitation wavelength between channels (nominally 450 nm, 590 nm and 650 nm), the chlorophyll fluorescence can also be mapped as a function of wavelength[1].

In his study, Goessling noted that in general terms his team was interested in better understanding how cells control

and optimize local conditions in order to maintain an optimum light environment for efficient photosynthesis. While low light intensities could limit photosynthesis, photo damage to the chloroplasts can occur when under high light irradiance. Phototrophic organisms use a variety of structures and sensing mechanisms to adapt and orient their components to maintain optimum illumination during natural variations in incident sunlight. The light environment is perceived at the most fundamental molecular level, by activation of signal cascades that result in selective activation of various transcription factors. For example, it is already known that chloroplast avoidance movement is primarily controlled by response to blue light, while red wavelengths trigger the reverse physiological response.

Goessling's team wanted to investigate if and how localized laser illumination could be used "to manipulate photosynthesis in single cells with spatial and temporal selectivity, while using chlorophyll fluorescence as a monitor of photosynthesis activity." In the experiments, this illumination was provided by a wavelength selectable laser source (a SuperK COMPACT supercontinuum laser from NKT Photonics in Denmark), which is coupled via a single mode fiber to channel the spectrally selected wavelength to the IS-OGP (Figure 3).

The laser light source was set to 650 nm and the focused spot directed at a single chloroplast in an Ananas comosus leaf cross section (Figure 4). As the accompanying graph shows, chlorophyll fluorescence yields are constant over the time duration of the experiment in chloroplasts distant to the laser illumination, while the chlorophyll fluorescence is quenched in chloroplasts close to the spot of laser illumination. After a short induction period, the chlorophyll fluorescence in the laser-illuminated area (denoted by the inset square in the images) steadily decreases. Goessling explained that this fluorescence quenching is due to the exciton energy being channeled instead into the photosynthetic electron transport chain. Interestingly, quenching of chlorophyll fluorescence is also observed in chloroplasts located in close proximity to the illuminated area, although they are not incidentally illuminated by the actinic laser light source. This observation might show scattering and redistribution of incident light by internal cellular structures in the plant cell, that is, the cellulose cell walls.

The capability to focus and manipulate a laser light source within a fluorescence microscope opens up numerous possible lines of research within the field of photosynthesis. Because the laser spot can be focused to a diameter under 10 µm, Goessling noted that it could be used to study microscale processes such as the light-induced movement of single chloroplasts. It can also illuminate a single guard cell, allowing researchers to study the influence of different wavelengths of light on stress signaling and light color-dependent stomata closure and opening.

In spite of its long history, researchers are still finding new ways to use the fluorescence microscope. These applications often require modifying its optomechanical capabilities.

Meet the author

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Reference

1. E. Trampe, et al. (2011). Rapid assessment of different oxygenic phototrophs and single-cell photosynthesis with multicolor variable chlorophyll fluorescence imaging. *Mar Biol*, Vol. 158, pp. 1667-1675.

A Key Term in This Article

• **Epifluorescence microscopy**: Fluorescence microscopy in which the excitation light comes from above the sample. Fluorescence was first done in transmitted light but epifluorescence proved more efficient, since the excitation light only needs to pass through a thin coverslip. The excitation light illuminates the sample and the resulting fluorescence is collected by the objective, narrowly adjusted by a filter cube, and is then sent to the detector. Epifluorescence has been the dominate design for microscopy since the early 1980s.

See **EDU.Photonics.com** for this and other definitions in the *Photonics Dictionary* and more information in the *Photonics Handbook*.