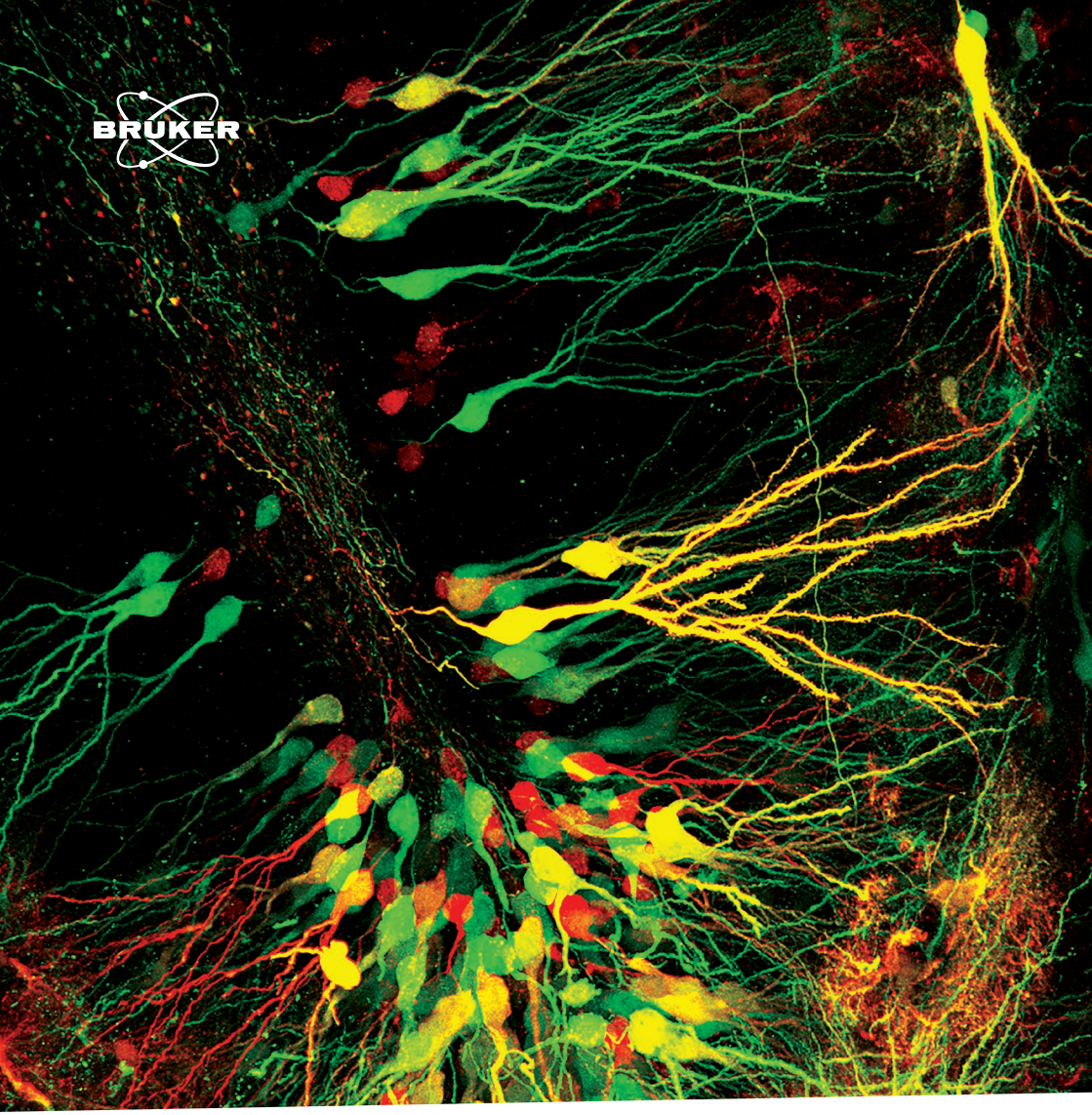


FLUORESCENCE MICROSCOPY

Multiphoton Microscopy

2nd EDITION

See Deeper into Brain Activity

Acknowledgments

Portions of this book were sourced with an Essential Knowledge Briefing from John Wiley & Sons Ltd in 2018 and with data and images originally published by Bruker customers. We thank them for their support and excellent research.

Cover Image:

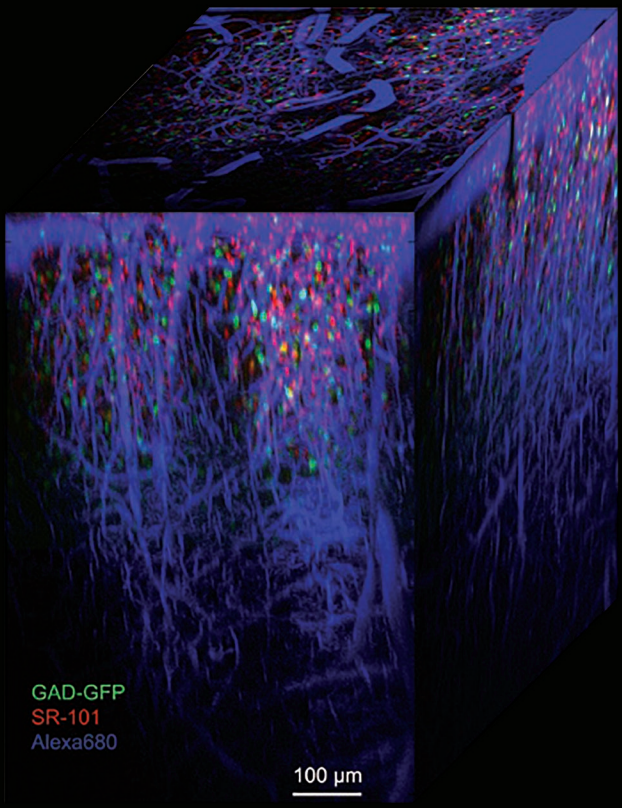
Two-color hippocampal brain slices taken on the Ultima 2Pplus. Image courtesy of Bryan Luikart, University of Alabama Heersink School of Medicine.

Introduction

Modern light microscopy was born with the invention of the fluorescence microscope in the early 1900s. Since then, it has been used to make important discoveries, such as visualizing animal and plant tissues for the first time, and now is leading breakthrough discoveries in various scientific disciplines. Fluorescent light microscopy works by labeling specimens with fluorescent tags, or fluorophores, that emit visible light when excited with light at other wavelengths. However, out-of-focus flare and limited sensitivity were limitations that led to the widespread adoption of confocal microscopy in the late 1980s. Confocal microscopes are popular for generating thin optical sections by focusing a laser beam at one depth level at a time and using a pinhole to block the out-of-focus flare. Nevertheless, confocal microscopes struggle with how deeply and quickly they can image inside a sample, which has spurred the development of multiphoton microscopy—a more powerful fluorescent microscopy technique that can produce three-dimensional (3D) images of biological structures and processes at unprecedented depths.

The most widely used form of multiphoton microscopy is two-photon (2P) microscopy. By combining two photons with half the energy to excite fluorophores in a specimen, 2P microscopy can reveal dynamic biological processes deep within living tissue with minimal damaging effects from photobleaching. This technology evolved from confocal microscopy, which has a similar principle but only one photon is absorbed by a fluorophore (in a linear interaction), causing it to emit a single fluorescent photon. Furthermore, three-photon (3P) imaging involves the simultaneous absorption of three photons – each with a third of the energy or three times the wavelength of the single photon. Using these longer wavelengths offers even deeper imaging capabilities and allows non-invasive recordings of neuronal activity at high resolution. Lastly, advanced features such as modulated focus are enabling almost instantaneous imaging in different planes.

This book introduces multiphoton techniques in the context of life science research. It reviews in detail the working core components of a modern multiphoton microscope followed by case studies where Bruker's Ultima multiphoton imaging technology was used to explore a variety of diverse research with organoids, astrocytes, brain slices, and optogenetic stimulation in different biological systems. Finally, there is a discussion about how these innovative technologies overcome various difficulties of performing cutting-edge research with fluorescent microscopy and looks toward recent technological advancements. Such advancements include measuring electrical impulses across the brain and are evolving with future research trends.



In vivo two-photon imaging through an optically transparent electrode array. Three-dimensional projection of a two-photon image stack. The vasculature is visualized by intravascular injection of Alexa 680-labeled dextran. Inhibitory neurons are labeled with eGFP. Astrocytes are labeled with SR101.
Image courtesy of Anna Devor and Shadi Dayeh, University of California San Diego, USA.

Principles and Techniques

Core components

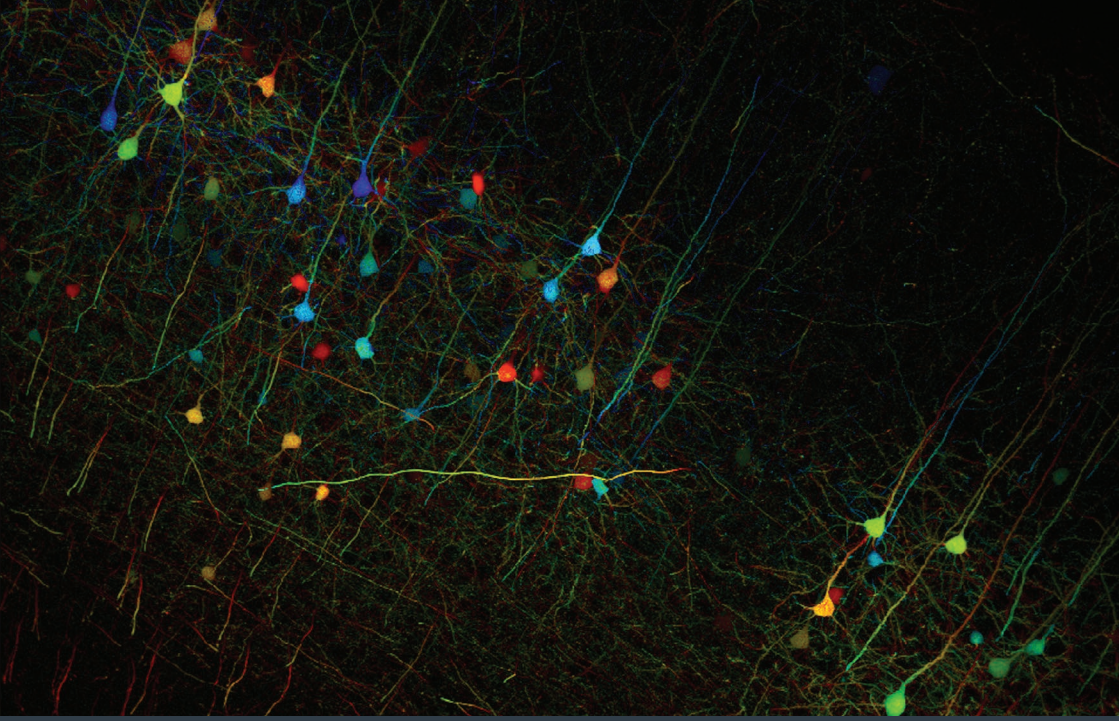
All multiphoton microscopes have several core components, although the precise specification may vary depending on the application. These core components include a femtosecond laser to produce a high-power photon beam, a scanning system to scan the focal point of the beam and create the image pixel-by-pixel, an objective lens, normally with a high numerical aperture, and a detector.

Lasers

The advent of ultra-fast pulsed (mode-locked) lasers was the main impetus behind the widespread adoption of multiphoton microscopes for life-science applications. The laser source must be high-power and mode-locked to create the photon concentration needed for two-photon absorption at the focal point. The most widely used tunable ultra-fast mode-locked lasers are Ti:sapphire types. These are able to produce femtosecond pulses, while being able to be tuned from wavelengths of 700nm to 1100nm, with some more modern versions being tunable from 660 to 1320. However, fiber lasers have become more popular as standalone lasers as a pump source for modern tunable lasers. Fiber lasers can produce powerful beams at high repetition rates and with femtosecond pulses but are limited by their fixed wavelength. Still, for many current two-photon applications in bioscience, only a few wavelengths are typically utilized, making them broadly useful today.

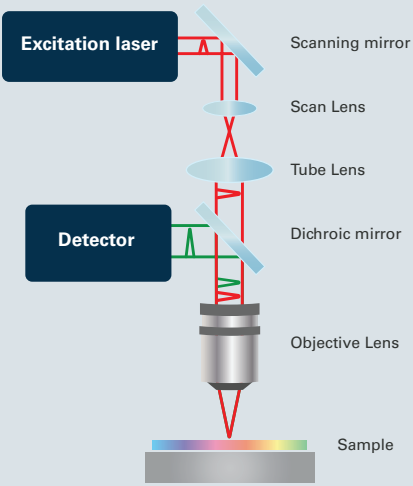
Scanning system

Two-photon microscopy relies on exciting fluorophores within a single focal point at a time. So, to build an entire 3D image, this focal point must be scanned over the sample with each focal point creating a single image pixel. Electrically controlled, moveable mirrors known as galvanometric (galvo) mirrors are typically used to scan the beam. With standard scanning galvo mirrors, the mirror angle is varied by motors. By having two mirrors for the X and Y planes, it becomes possible to create any arbitrary beam path.



Brain slice image taken on the Ultima Investigator Plus.

Multiphoton Core Components



A schematic of a scanning two-photon microscope, showing excitation by infrared laser pulses and collection of the resulting visible fluorescence or phosphorescence.

This is useful for various applications, such as tracing the outline of a cell membrane. The disadvantage of scanning galvo mirrors, however, is the limited speed at which they can move, due to heating of the motors caused by repeated acceleration and deceleration.

Resonant galvo scanner

A more recent development is the incorporation of a resonant galvo mirror into multiphoton systems. Resonant mirrors rotate or oscillate at a fixed frequency controlled by an electrical input. The beam is then scanned across the sample at this resonant frequency, allowing resonant galvo mirrors to create an image far faster than scanning galvo mirrors. However, resonant galvo mirrors do not illuminate the sample as evenly because the resonant mirror moves at different speeds during the scanning process. Some MPM systems offer both sets of scanners, providing the option of slower but more even scanning with standard scanning galvos or faster scanning with resonant galvos. More recently, techniques that go beyond simple point scanning have emerged that allow for faster scanning of samples. Methods to stimulate more than one spot are now made possible by using, for example, a spatial light modulator (SLM) to generate multiple spots that are swept across a sample simultaneously. Using multiple points will accelerate the collection of data but at the expense of the optical exclusivity that a single point benefits from. In the case where multiple points or frames are collected simultaneously, it can be difficult to uncouple the photon from the excitation source in the sample. In practice, this means that photons are now emerging from multiple areas at the same time, and photomultiplier tubes (PMTs) are agnostic to the source of any photon they report. Therefore, either computational methods to deconvolve the source of a photon are utilized, or two-dimensional sensors like cameras are used instead of PMTs in order to generate an image. Additionally, optical methods to increase the speed of raster scanning generated from resonant and galvo mirrors can also be implemented to increase the speed at which samples are swept, in both in XY planes as well as in Z depth.

Lenses

The objective and relay lenses focus the laser beam down to the focal point required for imaging and focus the resultant emitted photons toward the detector. The numerical aperture of a lens and the wavelength of light used to image a sample impose the theoretical resolution limit of the microscope. The objective lens

also has a defined field of view, which determines the maximum angle at which scattered photons from the sample can still be detected, and hence the area the lens can image. Lenses are continually being developed to offer a higher numerical aperture with an increased field of view, as well as to correct for various aberrations that can cause the focal point to become larger than its theoretical limit.

High numerical aperture air and water immersion lenses are often used with multiphoton microscopes. Water immersion, or dipping lenses, are prized due to their high numerical aperture and their ability to be used in conjunction with systems that require perfusion of solutions, which many in vivo and slice experiments require. Additionally, using a dipping lens reduces refraction-related issues, as there is only a single aqueous medium through which light must travel. In situations where a dipping lens would not be appropriate, such as when imagining larger distances from a sample, or when water might not be suitable as a medium, air objectives are often used, although they typically have a lower numerical aperture, as the different refractive indices between air water and glass prevent efficient collection of light.

Detectors

The detectors most commonly used in multiphoton microscopy are photomultiplier tubes, which are highly sensitive detectors of light. The geometry of the detectors is a particular advantage of MPM over confocal microscopy. In confocal microscopy, it is essential that the excitation photons and the photons emitted from the sample follow identical paths. If the paths were different, it would not be possible to assign the photons to a specific pixel, as they could have come from other parts of the sample. This type of light path, where the excitation and emission light path are identical, is known as descanned. The disadvantage of a descanned geometry is that photons are lost each time they pass through mirrors or other optical components, and any photons that came from the focal point scattered will be lost.

With multiphoton microscopy, as all the emitted photons can only come from the single focal point, they do not need to travel along the same optic path to the detector to determine which pixel they belong to. This means one or more detectors can be placed anywhere behind the objective lens pupil. Known as a non-descanned configuration, this allows for more efficient

photon collection because the detectors can now be placed much closer to the objective lens. Now, highly scattered photons can be collected, and photon loss is reduced as the emitted photons now pass through fewer optical components before reaching the detector. In this sense, multiphoton imaging is somewhat simpler in configuration when compared to classical confocal microscopy. This simpler, non-descanned configuration allows for more sensitivity when detecting the desired photons that are the end result of fluorescence, but this configuration also allows irrelevant photons entering the system to be pulled into the detection path. Therefore, multiphoton systems are sensitive to outside light, and microscopes often must be optically shielded from the outside world.

Spatial light modulator

The same process of two-photon excitation that allows for such precision and depth in imaging also applies to applications of photoactivation. That is if we can limit the functional space in which light can activate fluorophores, as is done with two-photon imaging, why not also similarly limit the spatial effects of a different laser source intended to activate or uncage neurotransmitter at specific sites on individual neurons?

The original Prairie Ultima multiphoton microscope combined two independently controlled light paths in order to simultaneously image and stimulate targeted populations of neurons. These paths allow for precise spatial and temporal control of neural activity using two-photon laser light to stimulate opsins or uncage bound molecules such as glutamate. Despite its powerful capabilities, dual-path systems are limited by their ability to only address single points at a time for stimulation. As control of ensembles of neurons is desirable when trying to recreate natural activity in the brain, further development of stimulation methods led to the adoption of spatial light modulators (SLMs) in combination with the secondary stimulation light path in order to project light in complex patterns across volumes of tissue.

SLMs are devices that manipulate the phase, intensity, or polarization of light in a spatially varying manner. In microscopy, they are typically implemented as a reflective surface with a varying number and density of pixels, in many ways similar to traditional liquid crystal displays. However, rather than simply altering the intensity of light, SLMs modify the wavefront of the incoming

(collimated) light beam, resulting in interference patterns that can be projected into three dimensions as a hologram.

Wavefront modification in SLM applications can be complex and varied, as each individual pixel on an SLM chip can independently modify incident light. Therefore, SLMs have been implemented for a variety of functions, including adaptive optics where they can correct for optical aberrations in real-time, in high-speed imaging, where multiple imaging points can be generated in three dimensions to increase the rate at which a plane or volume may be imaged. Or, in photoactivation experiments, the SLM can project multiple stimulation points across volumes with high precision and speed to activate opsins or to uncage bound chemicals such as glutamate.

Bruker microscopes such as the Ultima 2Pplus incorporate SLMs primarily as a method for photoactivation during raster-type two-photon imaging. The SLM is coupled to the photostimulation path, which can be independently controlled by its own set of galvo mirrors. This coupling allows for panning and spiraling of the generated hologram, allowing it to cover a larger area with more homogeneity while increasing opsin activation efficiency and reducing phototoxicity. SLM usage is often paired with electrotunable lens (ETL), a remote focusing device that allows for volumetric imaging independent of the position of the generated hologram, meaning that patterned illumination of cells or compartments can be achieved undisturbed by the imaging of multiple planes.

SLMs on Bruker systems have a wide range of applications for photostimulation and can be implemented across many different orders of magnitude in range. For example, hundreds of cells in the cortex can be simultaneously stimulated while imaging potentially thousands of neurons in near mesoscale-type experiments. When coupled with the correct objective, the same system can produce three-dimensional spots with the size and precision needed to activate single dendritic compartments across a single neuron. Therefore, activating different inputs directly or through chemical uncaging of glutamate. Overall, SLMs have revolutionized microscopy by providing versatile tools for controlling and manipulating light, enabling a wide range of advanced imaging and manipulation techniques with applications across neuroscience and biology in general.

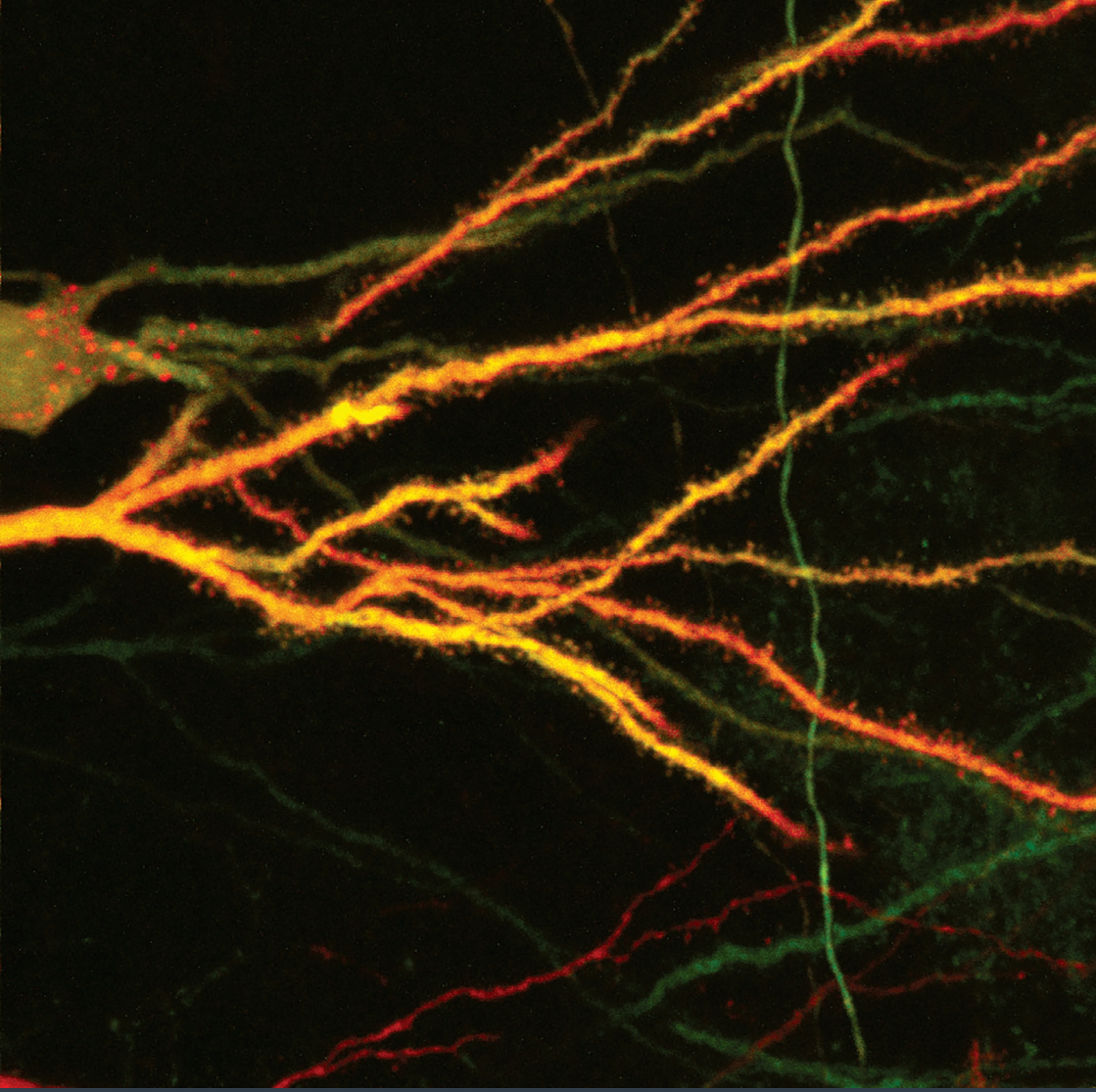
Electrotunable lens

Electrotunable lens (ETL) also known as liquid lens, is a variable remote focus device that varies focal length with electrical current. While traditional focus methods move the objective lens relative to a sample, an ETL alters the light path to change the focal length of the objective, thus changing the depth of the imaging plane. ETLs require only minimal physical changes to produce the desired effect on the focal plane and can therefore change imaging depth significantly faster than traditional methods. The lower inertial load that ETLs generate when focusing also allows for larger and arbitrary jumps in depth with little effect on the transition period, unlike focusing which involves moving the objective, where larger jumps are delayed by the greater inertial load of the lens. As this change in depth is independent of the position of the objective lens, this is considered a remote focusing device. Remote focusing is generally advantageous for experiments where optogenetics and imaging planes are uncoupled in depth.

Using a bare tunable lens introduces significant optical aberrations, therefore, Bruker combined the tunable lens with a relay lens system to reduce the aberrations. Bruker-designed drive electronics allow tunable lenses to be easily synchronized with other microscope components. On Ultima 2Pplus, the ETL is decoupled from the photostimulation light path and installed within the imaging resonant path. The ETL with a stationary objective enables imaging different planes while holographic 3D optogenetics or neurotransmitter uncaging experiments are performed on a multiphoton system. This arrangement is preferred over the use of piezo devices to move an objective lens between imaged planes to capture cell activity upon photostimulation.

Remote focus

Remote focusing in microscopy refers to any type of light manipulation that results in a change in imaging or stimulation depth without a change in the position of the microscope's objective. Some of the topics already discussed here either can be used as remote focusing devices (such as an SLM) or are expressly built for said purpose (such as an ETL). Other methods of focal manipulation exist as well. Acoustic-focusing devices, such as acousto-optic deflectors (AODs) can modify incoming light paths to remotely focus light. These operate on the acousto-optic effect, where waves



Two-color hippocampal brain slices showing spines, taken on the Ultima 2Pplus. Image courtesy of Bryan Luikart, University of Alabama Heersink School of Medicine.

traveling through a medium generate periodic variations in the refractive index of the material, thus allowing for light beams to be steered and modified with excellent temporal precision. Bessel beams can also be modified to act as a remote focusing device for fast Z-translation, or multiple-point illumination.

Bruker systems incorporate an ETL or a modified addressable Bessel-like beam for remote focusing using a custom-designed and controlled system that is fast, responsive, and flexible (see Electrotuneable lens in Principals and Techniques or DMD modulated focus in Future Trends for more detail). ETL are commonly used in conjunction with SLMs but can be used simply as a fast Z-device, or in conjunction with a traditional photo-stimulation path.

All of these remote-focusing devices are utilized for a single primary goal—to change focal planes independent of the objective's position. The main advantage to using remote focus occurs in instances where multiple light paths are intended to be used simultaneously on a sample without interfering with each other. In the most typical applications, this involves one laser input for imaging and a separate laser input for photoactivation (be it for optogenetics, uncaging, or other purposes). If traditional focusing methods are employed by either the imaging or the stimulation protocol, the other will inherently be impacted. For example, if one wanted to stimulate neurons in a single plane while imaging responses across a volume, moving the objective to image would interfere with the plane targeted for stimulation. In this case, using a remote focusing device for the imaging path allows the user to collect data from non-stimulation planes. Therefore remote-focusing components are essential to modern imaging-stimulation protocols utilized by many experiments.

DMD modulated focus

A recent advancement in remote focusing has been the addressable Bessel-like beam, where individual spots along the length of a beam can directly targeted in space using a digital micromirror device, or DMD. DMDs are controllable fast-switching arrays of tiny mirrors that can pattern light by changing the angles of light bouncing off of them. Because annular illumination of the back aperture of the objective produces the desired elongated Bessel-like beam, the size and thickness of the annulus can change the overall size and

position of the spot projected onto the sample with great speed and precision. DMD mirrors can switch in tens of microseconds, meaning that the delay in shifting spot location in Z is essentially instantaneous, meaning that there is no delay between collected frames, regardless of the size of the jump, as no lenses or optics are moving outside of the mirror array.

DMD-modulated focus is a remote focusing method, making it compatible with photostimulation protocols that require a stationary objective. Bruker microscopes incorporate this focusing type as an independent module, where it can act as a fast Z device, as well as an addressable Bessel-like beam with a controllable depth of field.

Bessel beams

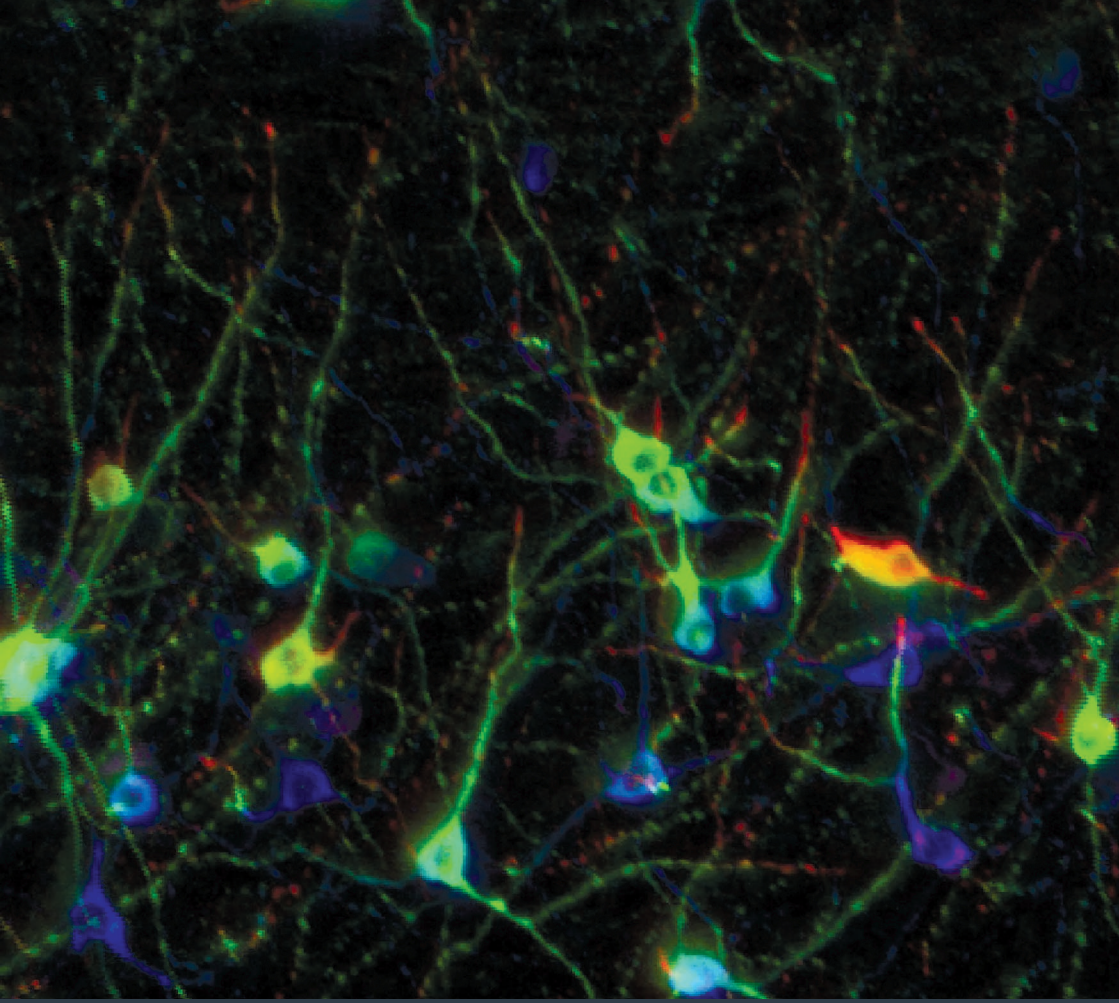
Bessel beams are a type of sample point illumination that produces reliable, homogenous activation spots that are elongated in the Z-axis when compared to traditional Gaussian-type activation spots. Laser-scanning microscopy methods, including two-photon microscopy, typically utilize a Gaussian beam which is straightforward to implement and is desirable for generating a small activation spot that can produce sharp images and tight focal planes. In contrast, Bessel beams are designed to illuminate thicker samples simultaneously when greater depth of field is required.

The concept of Bessel beams has its origins in the work of Friedrich Bessel in the 1800s and are generated in two-photon microscopy by illuminating the back aperture of the objective with an annulus instead of a uniform columnated beam (which produces a Gaussian spot). Bessel beams are typically generated by focusing light using an axicon rather than a traditional lens. Axicons are lenses with conical surfaces that generate the desired elongated and uniform spot that otherwise cannot be achieved using Gaussian beams. SLMs are also able to generate Bessel-like beams by altering the incoming wavefront to produce the desired interference. While an SLM can be more flexible than axicons, they are significantly more expensive and tend to be more complicated than the relatively simple and inexpensive axicon.

The main advantage Bessel beams hold over other illumination types is the extended depth of field they produce. The elongated focus of the beam allows for imaging larger scale features in Z without changing focal planes. Applications are typically in situations

where signals are sparse but cannot be captured by a single or even a few imaging planes. For example, imaging the entire dendritic field of even a single neuron, depending on the type, may require 100s of microns of imaging depth, requiring multiple planes to be imaged in order to collect the entire field. A Bessel beam could image the entire neuron in a single plane, as long as XY overlap is minimal in the sample. Similar situations occur if multiple layers of cells need to be imaged for an experiment, but the added time needed when imaging across planes reduces the temporal resolution of the experiment beyond what is needed. Therefore, the extended depth of field in certain situations can allow for much faster collection of data where temporal resolution and sample depth are of concern.

Bruker systems incorporate a modified Bessel-like beam that is addressable for customizable depth of field, as well as to act as an ultrafast remote focusing device.



Dopaminergic neurons of the mouse ventral tegmental area are color-coded by depth. 120µm section scanned using Bruker's NeuraLeap module.

Problems and Solutions

Live imaging of dynamic biological processes

One of the greatest challenges for multiphoton microscopy is imaging dynamic biological events, such as immune cell interactions or neuron firing, in real time since many occur over extremely short timescales. For any microscopy technique, capturing these dynamics requires imaging at high speeds without causing photodamage to the sample. Multiphoton microscopy benefits from using longer wavelengths which causes less photodamage to the sample surrounding the focal point as only fluorophores at the focal point of the beam are excited.

Imaging at rapid speeds requires delivering sufficient photons to the focal point in a short space of time and the ability to move the beam across the sample faster than the biological process being studied. Scanning the focal point across the sample quickly is currently achieved with resonant scanners. When operating a galvo mirror at a resonant frequency, images can be obtained at up to 40 frames per second, whereas scanning galvo mirrors are limited to 1.5 frames per second. Nevertheless, further speed increases are always being sought after and one avenue of research is the use of electrically tunable lenses, which allow the focal point to be scanned in the axial plane (See ETL in Principals and Techniques).

Image processing

There are many different forms of image processing fit for different applications. In the case of live in vivo imaging, users commonly need to correct for motion artifacts produced by the movement of the area being imaged between frames. Motion artifacts can be caused by animal movements, breathing, or even heartbeats. One must also account for subject drift where the sample being examined alters its position over time.

Good experimental design is the first step in correcting for motion artifacts and subject drifts. Many special experimental setups exist, such as head frames or kidney cups, to keep organs and organisms still during imaging but, some artifacts are hard to eliminate such as in awake animals. A common method to account for these artifacts is using a reference image. This works by choosing one image acquired during the experiment, either by the researcher or

a software package, so that other frames can be compared to this reference image and rejected if they are too dissimilar. Another strategy is to gate imaging during quiescence, or specific points in the breathing or cardiac cycle, by linking the multiphoton image capture software to other physiological recording devices, such as an electrocardiogram device. This is more commonly used in cardiac multiphoton imaging, as the heart cannot be completely immobilized. Specific image processing pipelines are important and used for cleared organs as this can generate single-cell resolution data sets for whole organs that are several terabytes in size.

Multicolor multiphoton

As with all fluorescent imaging techniques, the ability to image multiple fluorophores allows more sophisticated experiments to be performed. The fact that different fluorophores are excited by and emit slightly different wavelength photons can be used to distinguish multiple fluorophores within one sample and allow for selective excitation or labeling of specific structures or processes. In theory, different fluorophores can be distinguished by exciting only one fluorophore at a time through a tunable laser source or by exciting all fluorophores and filtering the emitted fluorescence for the wavelength of choice. In practice, fluorophores normally have overlapping spectra, particularly for two-photon excitation, as these peaks are generally very broad. Therefore, multicolor multiphoton imaging tends to employ a combination of different laser excitation wavelengths, emission filtering, and an analysis technique known as spectral demixing to separate the signals from individual fluorophores.

To maintain the imaging speed, multiple emission filters and a small number of excitation wavelengths are generally used. For example, two laser pulses can be used at wavelengths of 800nm and 940nm, with the emissions then detected by numerous detectors, each with a different filter. Non-descanned configurations with multiple detectors and filters make this type of setup possible and maximize the photons collected at each detector.

Fluorophore considerations

An important requirement when conducting experiments with multiphoton microscopy is to understand the spectral properties of the fluorophores being used. The two-photon cross-section of a fluorophore is a measure of two-photon absorption at different wavelengths and needs to be determined before a fluorophore can be used in two-photon microscopy.

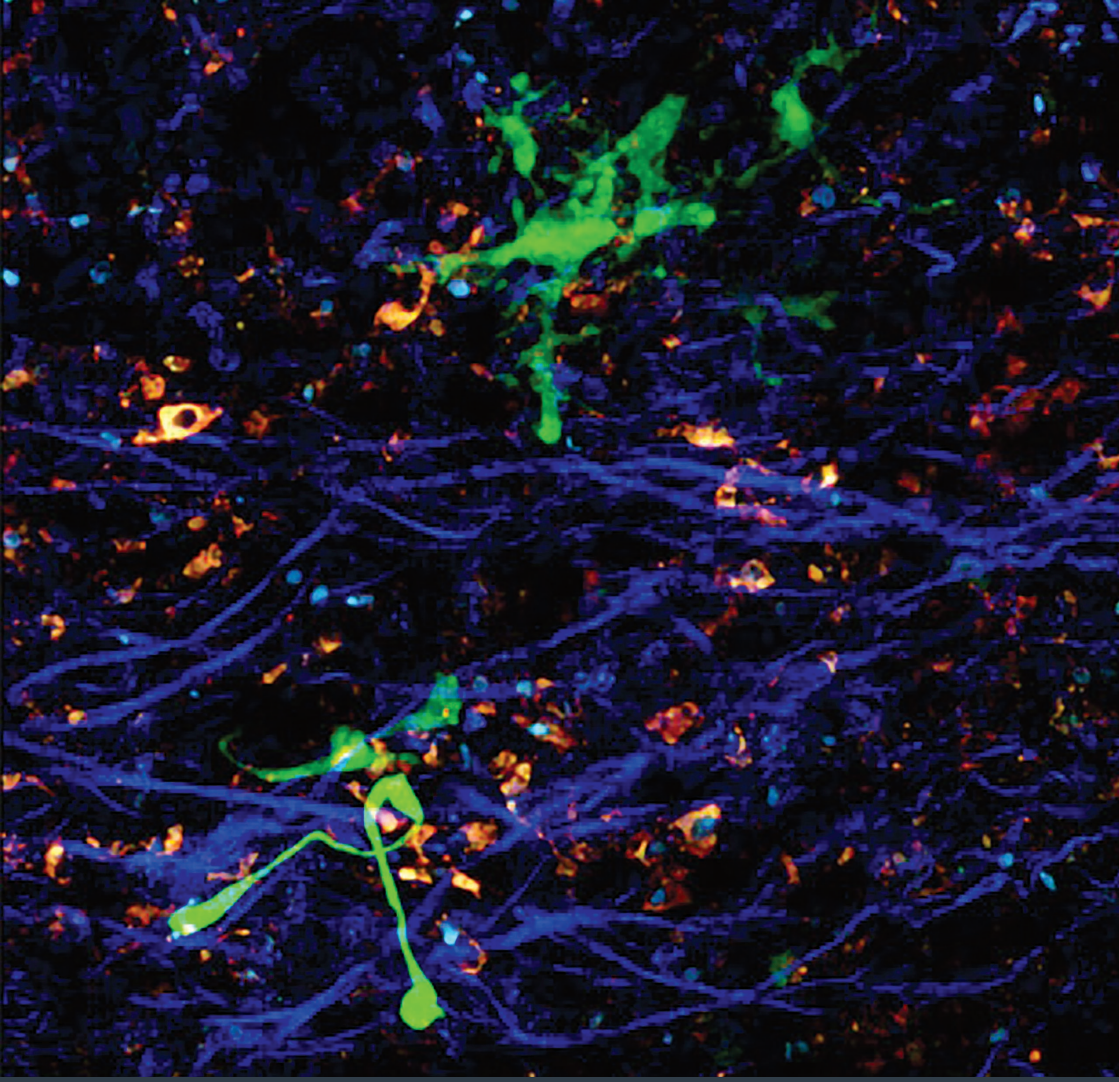
For two-photon microscopy, the proximity of the maximum cross-section of the fluorophore to the absorption peaks of water is an important consideration. Water makes up a high proportion of all tissues and has a broad absorption peak centered around 1500nm and beyond 1800nm. To prevent heating of the specimen, these wavelengths are generally avoided, and so two-photon cross-sections centered at 1300nm and 1700nm are often preferred for deep tissue penetration.

Simultaneous stimulation and imaging

Simultaneous optical stimulation and imaging offer huge potential for studying neural circuits. In various animal models, multiphoton microscopy offers the possibility of imaging active neural circuits with single-cell precision through the depth of the neocortex. Monitoring neuronal activity is possible using fluorescent microscopy alongside various markers. One specific and reliable marker is the genetically encoded calcium indicator GCaMP, a GFP-containing fusion protein.

Optogenetics is a technique for manipulating neural activity that relies on opsins, a family of naturally light-sensitive ion channels, which are found in the cell membrane of many microbes and allow the passage of different chemical species into or out of the cell in response to light. By genetically engineering neurons to express these proteins, they can be made to fire when illuminated with a particular wavelength of light.

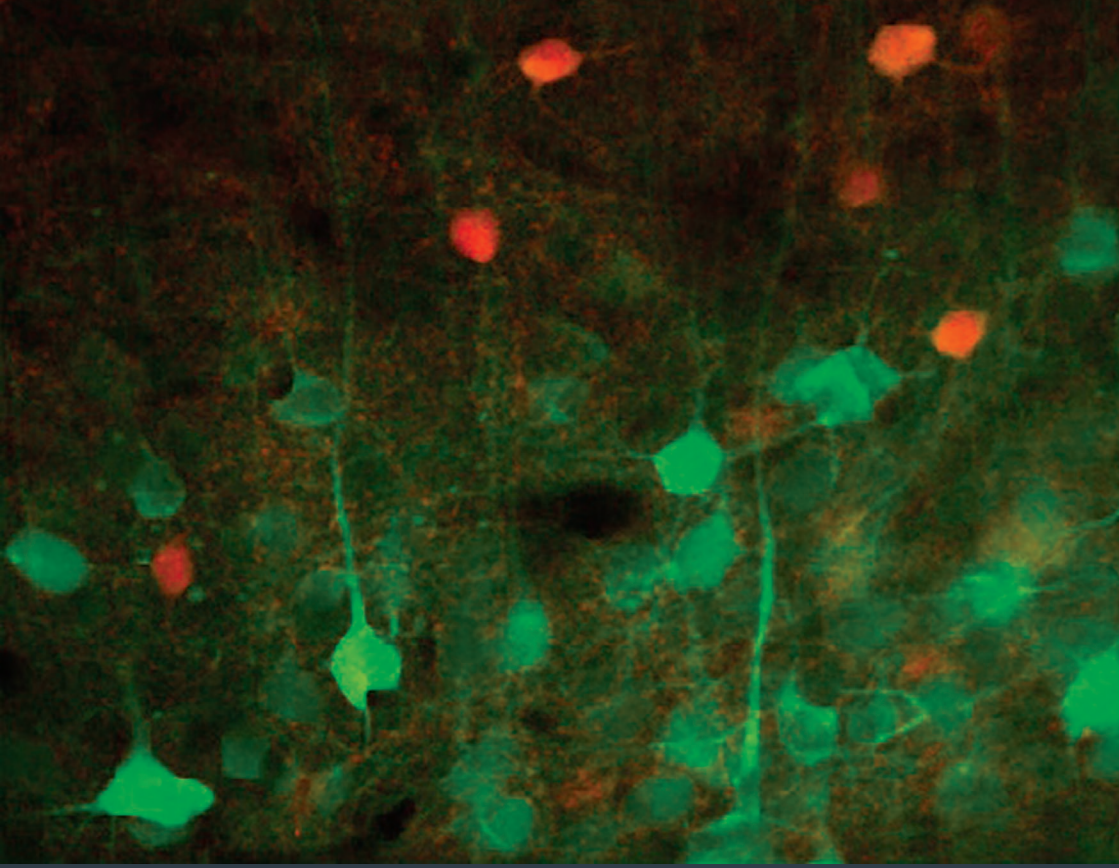
Neurons in animal models can be manipulated to express both opsins and GCaMP. When stimulated at one wavelength, the opsin channels open and cause the illuminated neuron to fire while the GCaMP calcium reporter can then be imaged via a second laser system. This results in neural activity being monitored in response to the specific activation. Furthermore, multiphoton microscopes



Two T cells (green) infected with a human immunodeficiency virus (HIV) amidst collagen fibers (blue, SHG) and autofluorescent cells (white/orange hues) in a lymph node.

that combine neuronal photostimulation and calcium imaging are now commercially available.

Other recent developments include using spatial light modulators (SLMs) to selectively excite ensembles of neurons in 3D within the brain whilst monitoring the response of all the neurons in the field of view. SLMs work by splitting a single laser beam into a number of spots in an arbitrary pattern. These single spots can then be scanned over a set of neurons to stimulate them. This technique has so far been used to stimulate hundreds of neurons simultaneously while monitoring the response of all the neurons in the field of view.



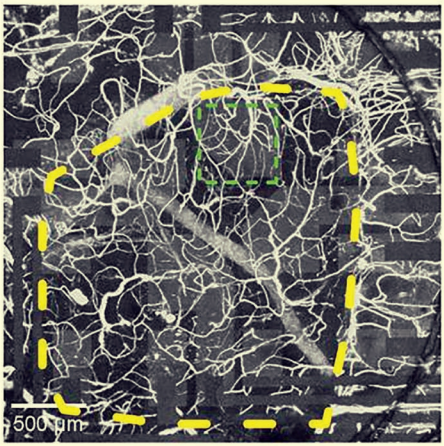
Chronic imaging through microprism in a live mouse, with interneurons labeled in red (td Tomato) and principal cells labeled in green (GCaMP6).
Image courtesy of Michael Goard, University of California Santa Barbara, USA.

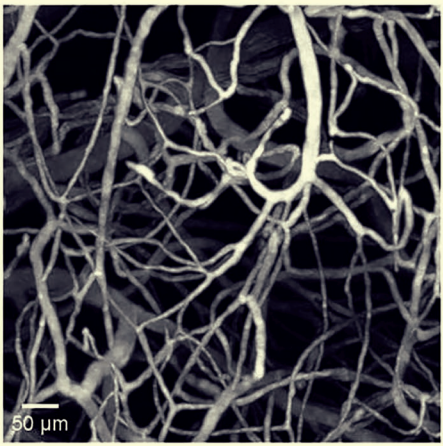
Applications

CASE STUDY

Multimodal monitoring of human cortical organoids implanted in mice reveal functional connection with visual cortex

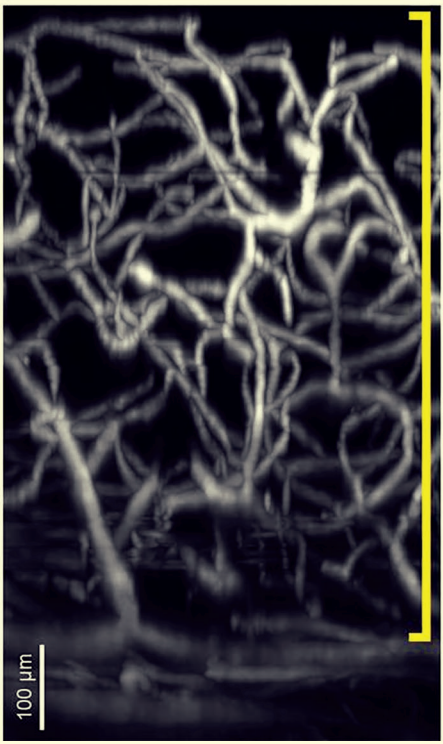
Authors Zihui Zhang, Lloyd E. Russell, Adam M. Packer, Oliver M. Gauldand, and Michael Häusser utilize an "all-optical" approach to perform in vivo imaging and simultaneously manipulate neural activity during behavior of an awake animal. Optogenetic interventions are targeted to individual neurons in the mammalian brain based on functional signatures recorded with the same microscope. The approach depends on two-photon imaging resolution and optical stimulation with a SLM alongside software that enables flexible targeting of readout and manipulation of visualized neurons. The ability to directly manipulate activity patterns using a closed-loop approach will enable improved tests of models of circuit connectivity, dynamics, and plasticity.





3D-view of volumetric stack recorded using the ETL focusing module. Layer 5B neurons in mouse visual cortex in vivo, labelled with tdTomato. Scale bar 100µm. Unpublished data (2019) courtesy of Lisa Bauer, Dustin Herrmann, Mehmet Fisek, Michael Häusser's lab, UCL, London.

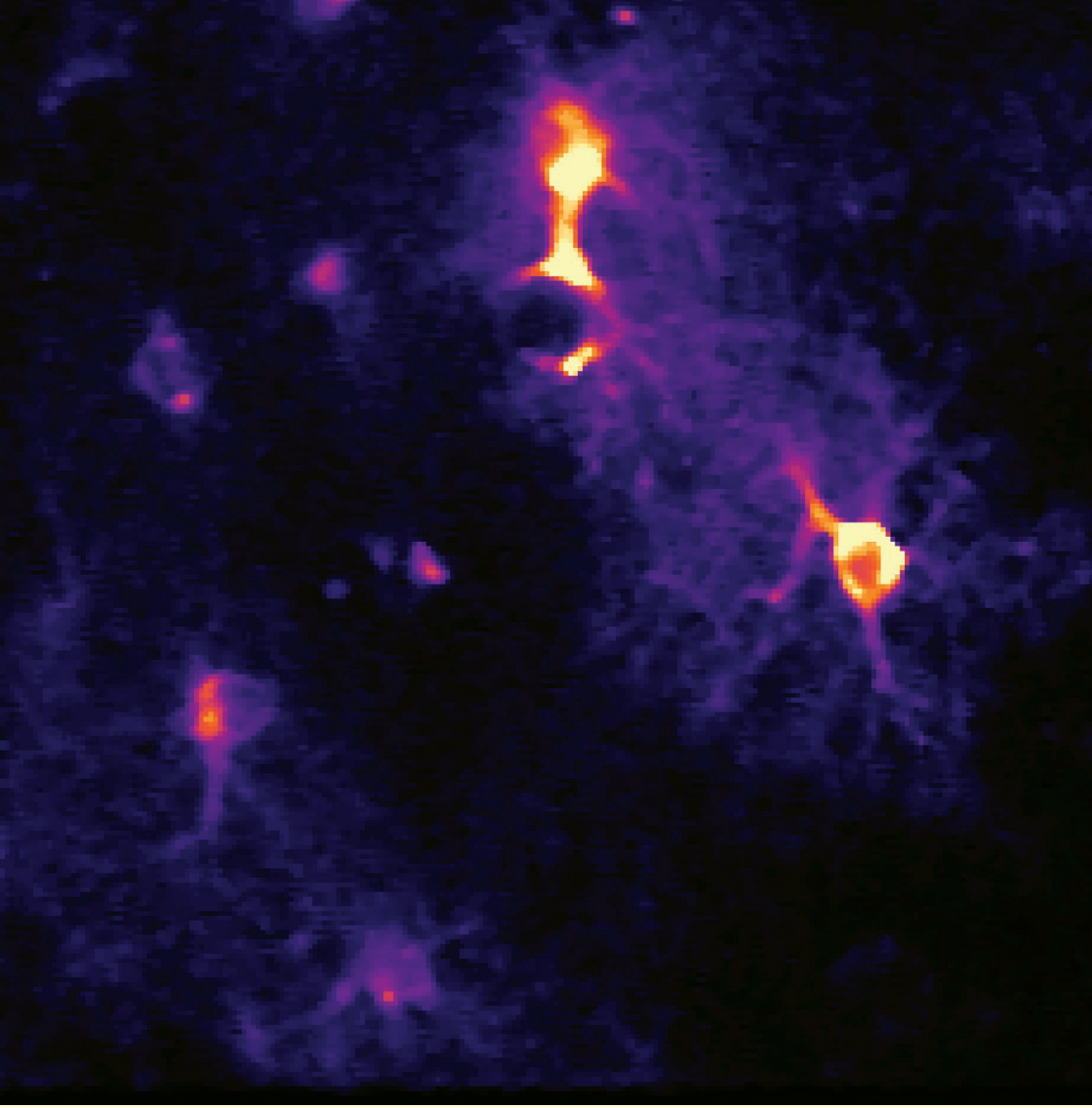
See Bruker's Research Highlight #2009 for more information.



CASE STUDY

A role for astroglial calcium in mammalian sleep and sleep regulation

Sleep plays a critical role in overall mammalian health, although many aspects of sleep regulation are not yet understood. Dr. Ingiosi, currently an Assistant Professor in the Department of Neuroscience at The Ohio State University, conducted research focused on the role of astrocytes, a type of glial cell in the brain, in sleep and sleep homeostasis. Using Bruker's Ultima Investigator multiphoton microscope alongside various tools such as electroencephalography (EEG) and miniscope imaging, she investigated how astrocytes interact with neurons to influence the sleep/wake behavior and which signaling pathways in the astrocytes are important. Dr. Ingiosi's research is well suited for continuing the investigation of the mammalian brain's role in sleep regulation.

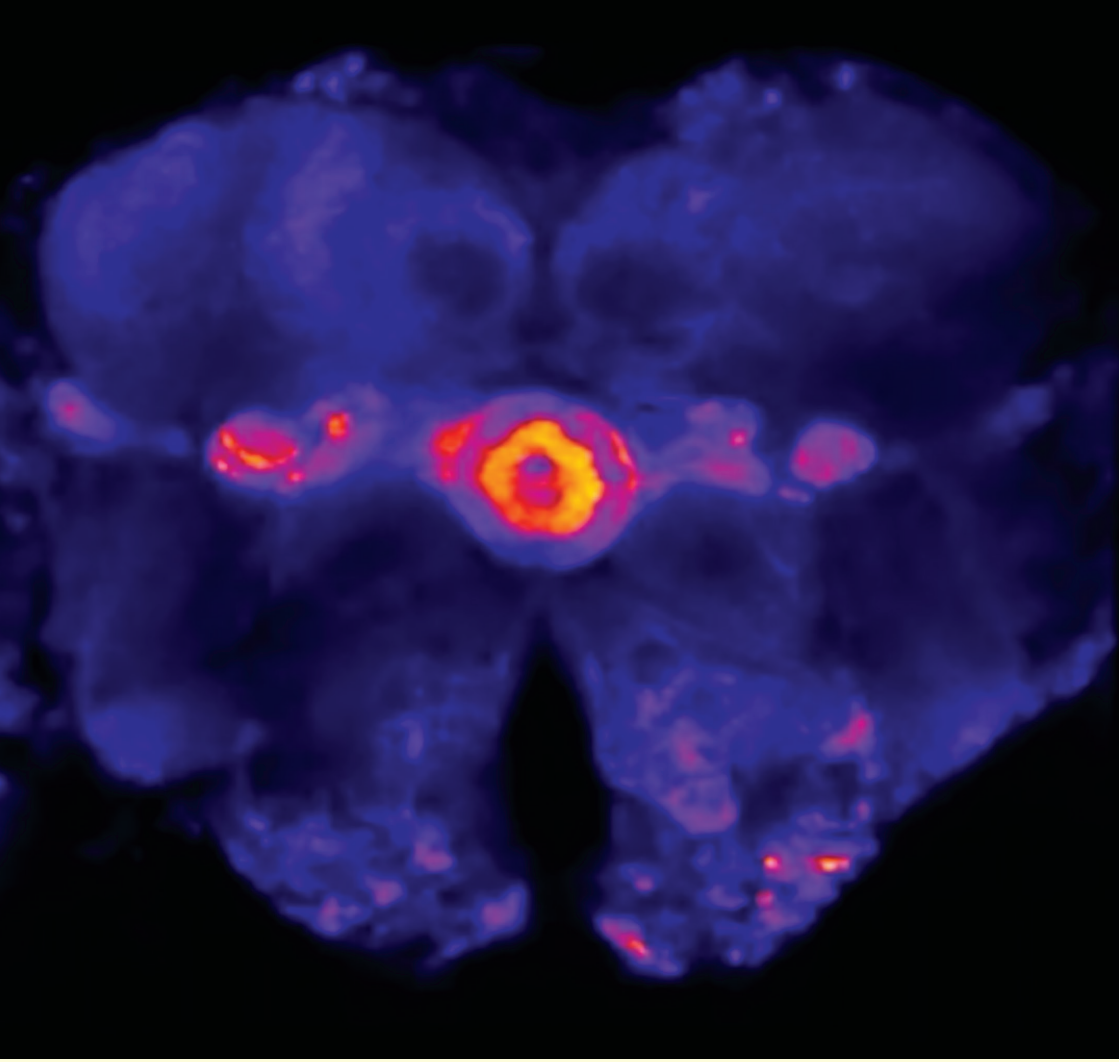


Two-photon image of astrocytes in the frontal cortex expressing the genetically encoded calcium indicator GCaMP6f.

CASE STUDY

Coupling between neural activity, metabolism, and behavior across the Drosophila brain

The brain is the most metabolically active organ in our bodies at rest, and Kevin Mann, Ph.D. investigated the relationship between neural activity and energy metabolism in the Drosophila brain. Using Bruker's Ultima two-photon microscope and local optogenetic perturbation, he was able to visualize the neural activity in Drosophila brains and conclude that metabolic networks exist and share structure with calcium networks in the fly brain. This work serves as an important platform for exploring the dependence of neuronal activity on metabolic flux in intact circuits on the timescale associated with behavior.

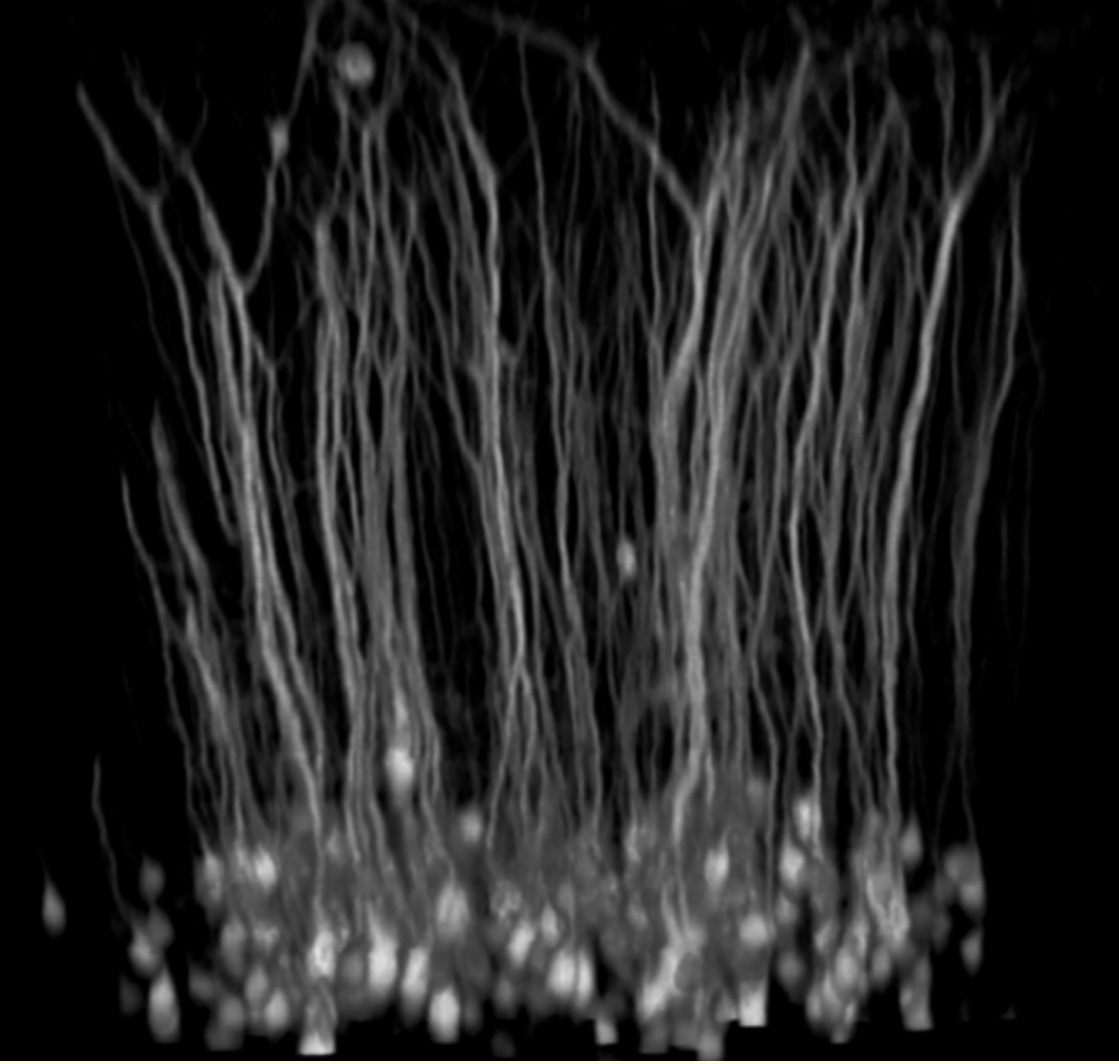


Scanned depth of an entire Drosophila brain pseudocolored to show spontaneous neural activity acquired with two-photon resonant imaging.

CASE STUDY

Closed-loop all-optical interrogation of neural circuits in vivo

Authors Zihui Zhang, Lloyd E. Russell, Adam M. Packer, Oliver M. Gauldand, and Michael Häusser utilize an "all-optical" approach to perform in vivo imaging and simultaneously manipulate neural activity during behavior of an awake animal. Optogenetic interventions are targeted to individual neurons in the mammalian brain based on functional signatures recorded with the same microscope. The approach depends on two-photon imaging resolution and optical stimulation with a SLM alongside software that enables flexible targeting of readout and manipulation of visualized neurons. The ability to directly manipulate activity patterns using a closed-loop approach will enable improved tests of models of circuit connectivity, dynamics, and plasticity.



3D-view of volumetric stack recorded using the ETL focusing module. Layer 5B neurons in mouse visual cortex in vivo, labelled with tdTomato. Scale bar 100µm. Unpublished data (2019) courtesy of Lisa Bauer, Dustin Herrmann, Mehmet Fisek, Michael Häusser's lab, UCL, London.

Recent Advancements

Voltage imaging

Voltage imaging is an emerging method in neuroscience where the brightness of a fluorescent indicator reports changes in cell membrane potential, rather than a second messenger, or other proxy such as neurotransmitter concentration. The majority of two-photon imaging experiments in neuroscience thus far have utilized calcium indicators as the main proxy for neural activity. These indicators have been developed over the years to have excellent sensitivity, signal-to-noise ratio (SNR), and a range of kinetic properties for faster or slower applications. Calcium activity is highly correlated with neural activity and therefore is a good proxy, but has limitations in its ability to detect sub-threshold events and can often be too slow to resolve many faster neuronal processes. Electrophysiology, in contrast, is a decades-old technique that can be used to directly measure membrane potentials and currents but is limited by the need to either physically couple with a neuron or have a recording device implanted in close proximity to a neuron or group of them. Therefore, there has been a concerted effort to develop voltage-sensitive indicators to allow for the direct optical measurement of voltage changes in populations of cells with less physical impact on the recording site.

Voltage imaging began with and is still to some degree performed with voltage-sensitive dyes. These indicators are dyes that insert themselves into membranes and change fluorescence based on the surrounding electrical potentials, thus being able to report changes in cellular membrane potentials with excellent spatiotemporal resolution. Dyes, while still utilized, can complicate experiments due to the inability to target them specifically to cell types and there are concerns with phototoxicity and the alteration of membrane properties by their very presence. More recently, efforts have been made to make genetically encoded voltage indicators (GEVIs) that function in a manner similar to genetically encoded calcium indicators (GECIs). In the case of most GECIs, the binding of calcium to an engineered fluorescent protein alters the overall fluorescent output of the molecule, thus changing brightness relative to local concentrations of calcium. GEVIs have utilized a similar concept, but rather than using a calcium binding domain, they are fused with the voltage-sensitive domains found in existing voltage-sensitive ion channels. The change in conformation due

to the shift of the voltage-sensitive domain changes the output brightness of the indicator, thus reporting changes in membrane potential as changes in brightness.

Voltage imaging, like calcium imaging, can be done with any sufficiently fast and sensitive microscope. As sampling rates approaching 1kHz are typically needed to resolve the types of signals, concerns such as dwell time and SNR arise as the ability to detect photons from fluorescent changes at such high rates becomes significantly more difficult. Therefore, current microscopes utilize methods to increase imaging rates. One example is with standard resonant-imaging systems, small windows can be sampled at 1kHz, but the field of view becomes significantly limited and restricted to elongated regions of interest. Methods for increasing scan rate allow for faster raster-type imaging but sacrifice overall signal due to reduced dwell time on samples. Solutions also have been demonstrated using SLMs to scan multiple illumination spots across samples but must use two-dimensional sensors such as CMOS chips over traditional photomultiplier tubes, as multiple illumination points cannot be distinguished.

Imaging and stimulating through GRIN lens and prisms

Multiphoton imaging, while excellent at penetrating tissue when compared to 1P imaging, is still fundamentally limited by tissue scattering or absorbing light. In cases where it is necessary to image deeper into tissue than can be normally accessed from the surface, gradient index lenses (GRIN) can be used to essentially project the imaging plane deeper into tissue. GRIN lenses are surgically implanted directly above the targeted tissue and allow the microscope to focus on a plane above the lens, which then refocuses light onto the sample. Therefore, otherwise inaccessible structures deeper in the brain can be imaged by implanting GRIN lenses directly into tissue at a desired location and depth. Common structures targeted are those such as the amygdala, the basal ganglia, or even brain stem regions where multiphoton imaging cannot otherwise access.

Similarly, imaging structures that cannot be accessed through cranial windows, or at angles not achievable due to other constraints, require the use of an implanted optical component—prisms. Prisms, much like GRIN lenses, are implanted into tissue at a desired location to allow optical access to otherwise

inaccessible regions. However, instead of focusing light, prisms reflect light at a specified angle in order to image tissue. A common example of the use of prisms is to image transverse sections of the hippocampus in vivo.

Both imaging and stimulation of cells through secondary optics like GRIN lenses and prisms is possible with multiphoton imaging. Modern lenses are compatible with common multiphoton wavelengths, (e.g., the Inscopix Proview™ 2P Lens), meaning that the same excellent spatiotemporal resolution expected from standard multiphoton imaging can be achieved with the depths and angles that these secondary optics provide. Holographic stimulation is also possible with current optics, albeit with somewhat reduced axial resolution due to the lower numerical apertures of GRIN lenses. Still, cellular resolution is achievable, and ensembles of neurons deep in the brain are accessible with Bruker imaging and SLM systems when combined with secondary optics.

Conclusion

Multiphoton microscopy enables deep, fast, and large-scale imaging for a variety of advanced biological research. Combining two or three photons with half or a third of the energy to excite fluorophores within the sample enables capturing dynamic biological processes with minimal photodamage. Furthermore, core components and additional modules, including ETL, SLM, and DMD focusing, support such applications as electrophysiology, all-optical interrogation, and the integration of human-derived structures into animal models for investigating various neurological disorders. Multiphoton microscopy is a powerful tool for functional imaging deeper into biological tissues than what is possible with confocal microscopy, and will continue to support innovative research in neuroscience and related fields.

Depth-colored projection of neurons in cleared-mouse brain slice.

Bruker Multiphoton Products

Ultima2Pplus Multiphoton Microscope

Ultima Investigator Plus Multiphoton Microscope

OptoVolt Module

NeuraLeap Digital Micromirror Device

NeuraLight 3D Ultra Spatial Light Modulator

Further Resources

Subscribe to the Fluorescence Microscopy Journal Club

Browse key publications and other multiphoton microscopy resources

Watch our Multiphoton Microscopy Webinars

Bruker Fluorescence Microscopy

Madison, WI • USA
Phone +1.608.662.0022
productinfo@bruker.com

See Deeper into Brain Activity

www.bruker.com/Multiphoton