
Scinapse
Useful guides for one-, two- and three-photon imaging experiments
Scientifica

A note from Scientifica

Scientifica provides world-class neuroscience equipment for electrophysiology, multiphoton imaging and optogenetics research.

This magazine contains guides to help you with your fluorescence and multiphoton imaging experiments. Each guide explains how the technique works, as well as when and why you would you use it and the limitations. We have also included a step-by-step guide for aligning your laser for two-photon imaging.

The guides included in this magazine can be found online at www.scientifica.uk.com. We have collated them here so they can be easily referenced in the lab.

Please get in touch if you have any feedback or suggestions, we would love to hear your thoughts via info@scientifica.uk.com.

Thank you
The team at Scientifica

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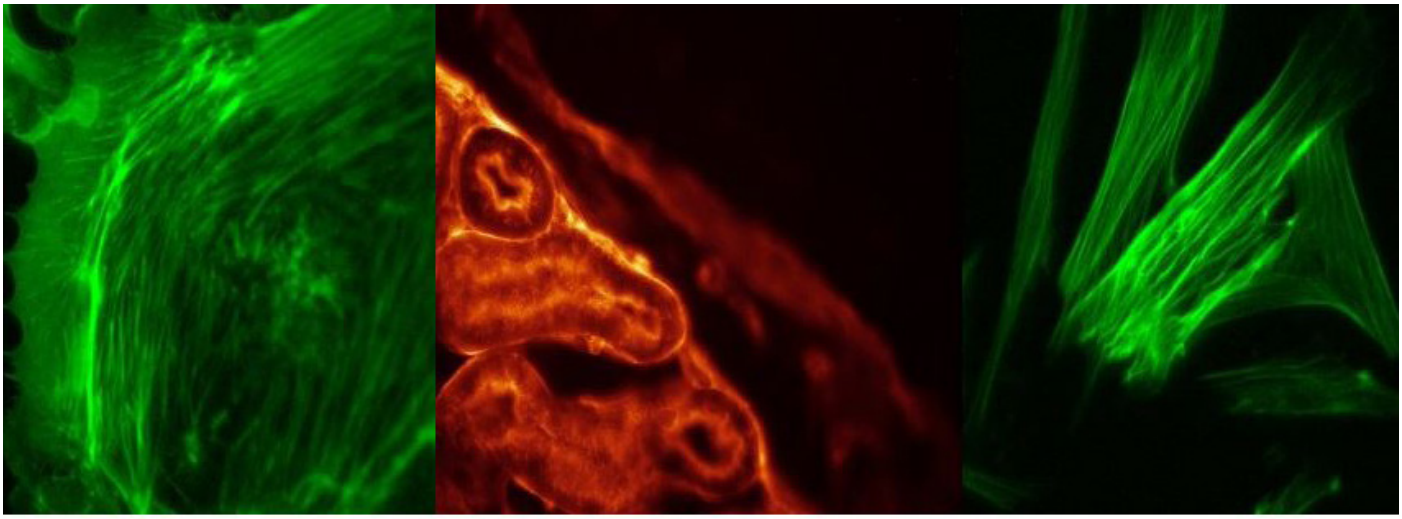


Widefield fluorescence microscopy

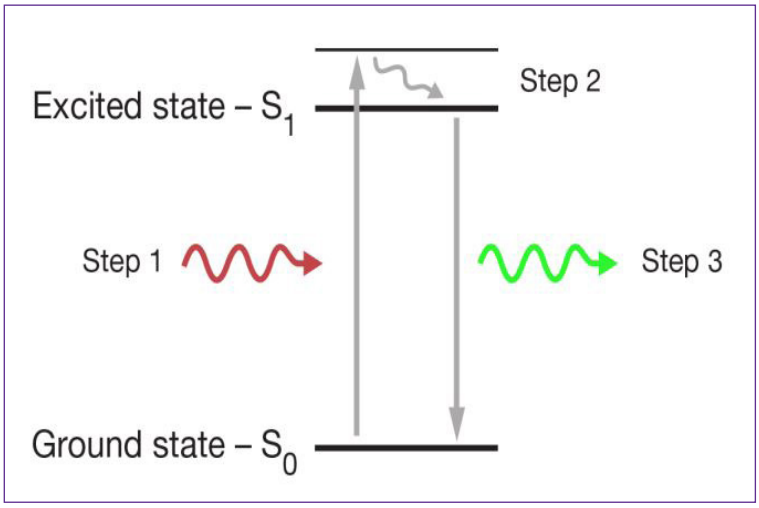
Widefield fluorescence microscopy is an imaging technique where the whole sample is illuminated with light of a specific wavelength, exciting fluorescent molecules within it. Emitted light is visualised through eye pieces or captured by a camera.

This technique enables the identification and visualisation of cells, cell components and defined proteins. Widefield microscopes are excellent for producing 2D images of specimens as the entire field can be captured at once. The amount and localisation of specific fluorescent molecules can be seen with fast temporal resolution. Processes in live cells, such as neuronal signaling, can be measured in real time.

Fluorophores

Fluorophores are molecules which can be excited by light of a specific wavelength (the excitation wavelength), causing them to emit light of a longer wavelength (the emission wavelength).

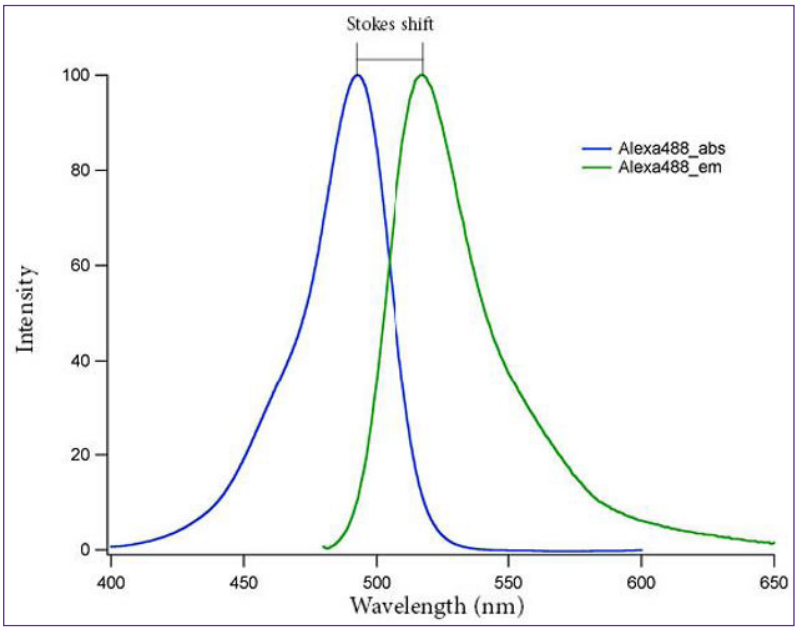
The light is absorbed by the fluorophore, exciting the electrons from their 'ground state' (lowest energy level) to an 'excited state' (higher energy level). When the electrons return to their ground state, they emit light of a longer wavelength, which is seen as fluorescence.



A Jablonski diagram demonstrating light exciting electrons within a fluorophore to a higher energy level, and the electrons returning to their ground state with the emission of light.
Step 1: *Absorption of a photon of light by electrons in the fluorophore, exciting them to a higher energy state.*
Step 2: *Vibrational relaxation, no emission of visible light.*
Step 3: *Electrons return to their ground state with the emission of a fluorescent photon.*

The difference between the excitation and emission wavelengths of a fluorophore is called the 'Stokes shift' (see graph). The range of wavelengths, and thus colours of light, that a fluorophore can absorb and emit are known as excitation and emission spectra. All fluorophores have peak wavelengths of excitation and emission. This difference in the excitation and emission spectra of individual fluorophores means they can be used to differentiate separate targets in the same sample.

Fluorophores can be used to label specific parts of biological samples. They can be naturally occurring in organisms, such as chlorophyll. They can also be introduced into organisms through genetically engineering their DNA to encode fluorescent molecules. Alternatively, antibodies can be tagged with small molecule fluorophores, such as Texas Red, and then introduced into the specimen.



A graph showing the Stokes shift of GFP

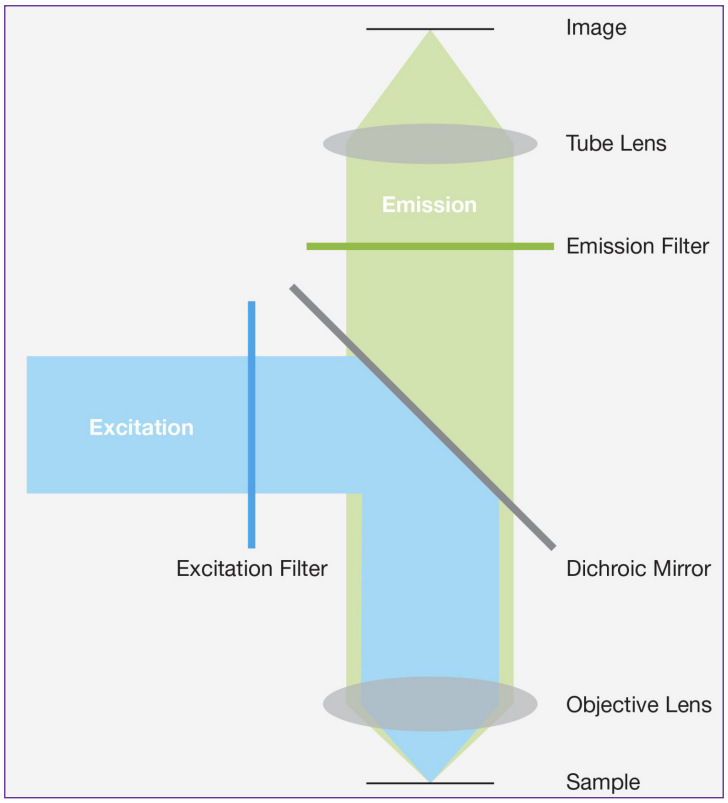
Fluorophores emitting colours across the visible light spectrum have been developed. Commonly used fluorophores include Green Fluorescent Protein (green emission), 4',6-diamidino-2-phenylindole – DAPI (blue emission) and Texas Red (red emission).

How fluorescent microscopes work

Excitation light is emitted from a light source and passes through an excitation filter which only allows light of specific wavelengths that excite the fluorophore to pass through. This excitation light is reflected off a dichroic mirror, focused through the objective lens and onto the sample.

When the excitation light hits the sample, fluorescent molecules within the sample are temporarily excited to a higher energy state. When they relax back down to their ground-state, light of a longer wavelength is emitted.

Some of the emitted light is collected by the objective lens, transmitted by the dichroic mirror then passed through an emission filter. The emission filter blocks excitation light and transmits emission light to the eyepiece/camera to be viewed or imaged.



Demonstration of how a widefield fluorescent microscope uses light to image a biological sample containing fluorescent molecules.

This technique, where both the excitation and emitted light pass through the same objective lens, is epifluorescence – 'epi' is derived from the Greek meaning of 'same'.

The excitation filters and dichroic mirror are usually located in a filter cube. By only allowing light of specific wavelengths to pass, the filter cube reduces the 'noise' from the sample, ensuring a clear image is produced which only shows the fluorescence of specific fluorophores.

The light source

The most common light sources used today are light-emitting diodes (LEDs). The wavelengths and intensities of light they produce can be precisely selected and controlled, they are inexpensive, do not produce excess heat, do not require alignment and are very compact. These properties make them a superior light source to use in comparison to arc-lamps and tungsten-halogen lamps which were commonly used in the past.

Mercury or xenon arc-lamps have powerful intensities at set wavelengths, however they produce a lot of excess heat, have a high intensity (so photobleaching and phototoxicity are more likely) and have to be disposed of as hazardous waste due to the nature of the elements they contain. They also have a much shorter lifetime than LEDs.

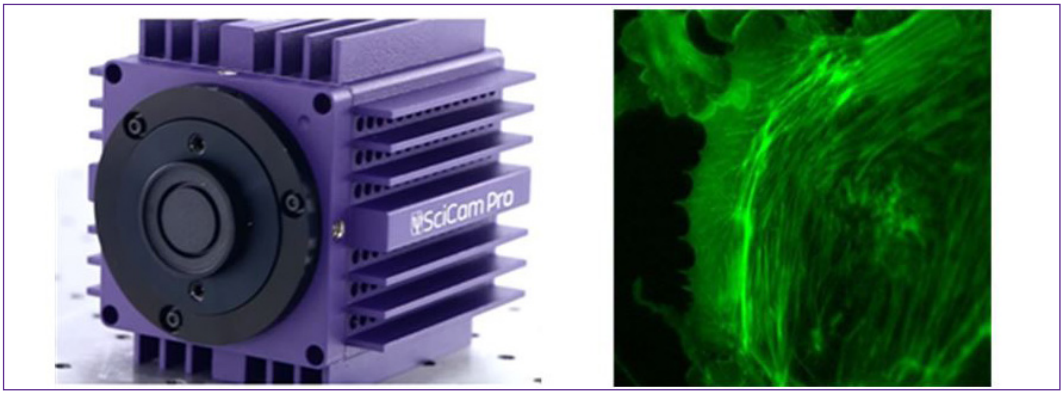
Tungsten-halogen lamps have a reduced phototoxicity, are less expensive and provide a more even illumination compared to arc-lamps. However, their intensity is lower and can be too low to image weak fluorophores.

The camera

Samples can be observed using an eyepiece, however, a camera is needed for image acquisition. The cameras contain millions of photodiodes, which each have semi-conductor sensors that convert light into electrical current.

The most common semi-conductor sensors are CCD (Charge Coupled Devices) and CMOS (Complementary Metal Oxide Semiconductors). The camera used depends on the required frame-rate, noise level and sensitivity.

sCMOS (scientific CMOS) cameras are also used. These offer low noise, rapid frame rates, wide dynamic range, high resolution and a large field of view simultaneously. sCMOS cameras are suitable for high fidelity, quantitative research and low-level light environments.



Left: the highly sensitive Scientifica SciCam Pro CCD camera.
Right: a Bovine Pulmonary Artery captured by the SciCam Pro using widefield fluorescence microscopy.

Electron Multiplied CCD cameras enable fast detection of low-light fluorescence. Cooled CCD cameras allow fluorescence signal to be gradually accumulated with little noise while maintaining high resolution. Both developments allow for faster imaging with better contrast at low signal levels.

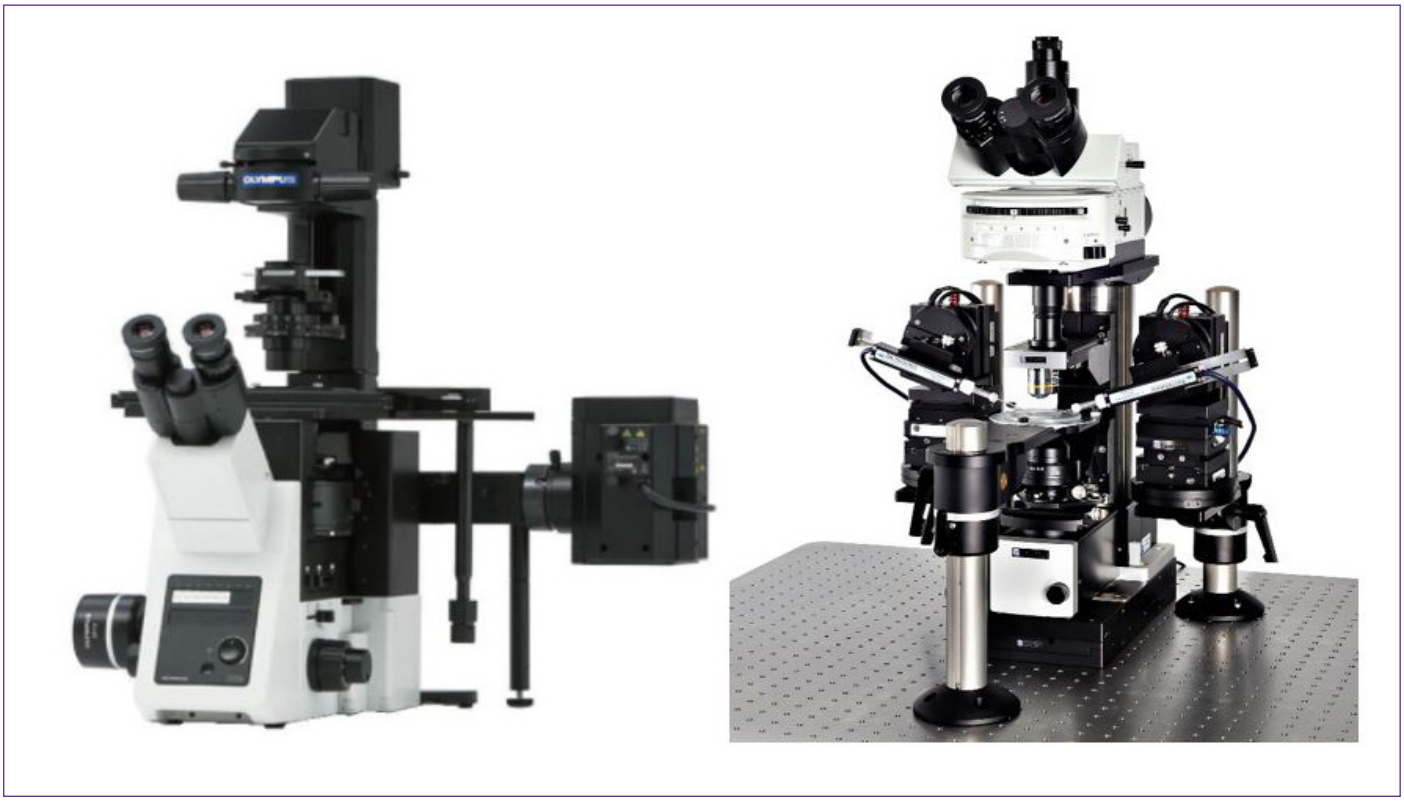
Resolution

Widefield microscopy can produce high-resolution images. However, because the whole sample is illuminated, there are factors that can reduce the resolution. It can be difficult to tell how deep in the sample the fluorescence originated from, especially in thick samples such as live cells or tissues, as the emission light is incorporated throughout the sample. The emitted fluorescence can scatter, making the image appear blurry. This makes widefield microscopy sometimes unsuitable for producing 3D images.

Microscope configuration

The sample can be illuminated from above, using an upright microscope, or from below, using a microscope in an inverted configuration.

Inverted microscopes are best for viewing living cells in culture, whereas upright microscopes are more suitable for viewing fixed samples such as tissue slices.



Left: Olympus IX73 Inverted Microscope, supplied by Scientifica, which illuminates samples from above. Right: Scientifica SliceScope Pro 1000 system which illuminates samples from below.

Two-photon microscopy: Why two is better than one

Two-photon microscopy is a fluorescence imaging technique that allows the visualisation of living tissue at depths unachievable with conventional (one-photon) fluorescence or confocal microscopy.

Also known as non-linear multiphoton, two-photon laser scanning or simply two-photon microscopy, it relies upon the principle of two-photon absorption.

How two-photon microscopy works

Two-photon absorption is the concept that two photons of identical or different frequencies can excite a molecule from one energy state (usually the ground state) to a higher energy state in a single quantum event.

This state can be described physically as the jump of an electron from one atomic orbital to another that is less stable. The energy difference between the two states is equal to the sum of the energies of the two-photons absorbed.

The two photons must hit the molecule within 1 femtosecond of each other (10-15 seconds). This requires a focused laser that is able to produce very fast pulses of light (~80 MHz) with a lot of power (~150,000 W peak power).

In two-photon microscopy, two photons of light, with double the wavelength of the single photon used in traditional fluorescence microscopy, are used to excite the same or similar fluorescent dyes. However, only one photon is released when the electron drops down to its more stable orbital. This will have the same wavelength as the equivalent one-photon fluorescence method (i.e. slightly longer than half the excitation wavelength).

The most commonly used fluorescent dyes have excitation spectra in the 400 to 500 nm range. The wavelengths used to excite the same dyes with two-photons therefore tend to be between approximately 800 and 1000 nm, in the infrared spectrum.

Why it is useful for imaging living tissues

A key benefit of two-photon microscopy is its ability to restrict excitation to a tiny focal volume in thick samples. The objective focal point is the only space with a high enough photon density to ensure simultaneous presentation of two photons to the fluorophore. Effectively, this means there is no out of focus emission light and any light at the emission wavelength must have come from that single spot.

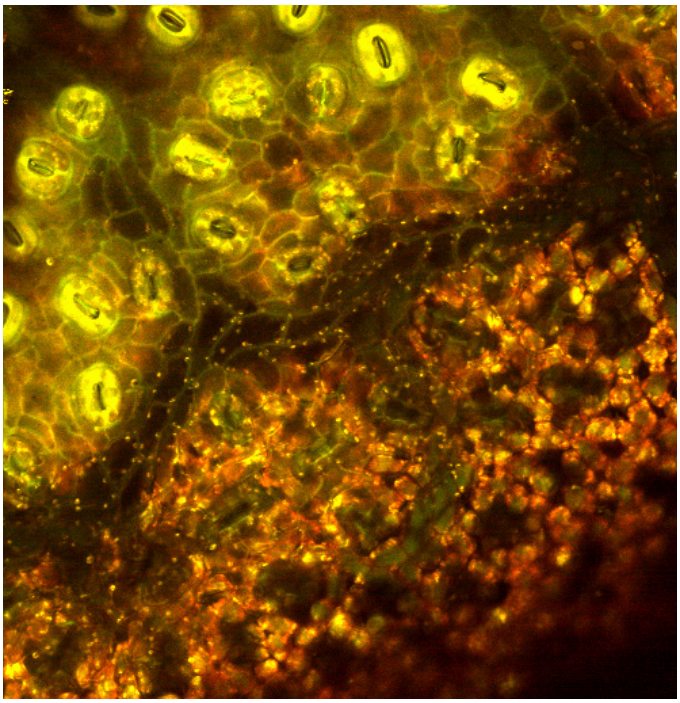
By scanning the laser over the region of interest it is possible to build up a 2D image of the fluorescence occurring

in the living tissues. Carrying this out at various focal planes enables the formation of a high-resolution 3D model. Collected over time, these images or models can be used as the frame of a movie to visualise processes as they happen. The temporal resolution is limited by the scanning speed of the mirrors and the amount of time it takes to build up a significant fluorescent signal at each pixel (dwell time).

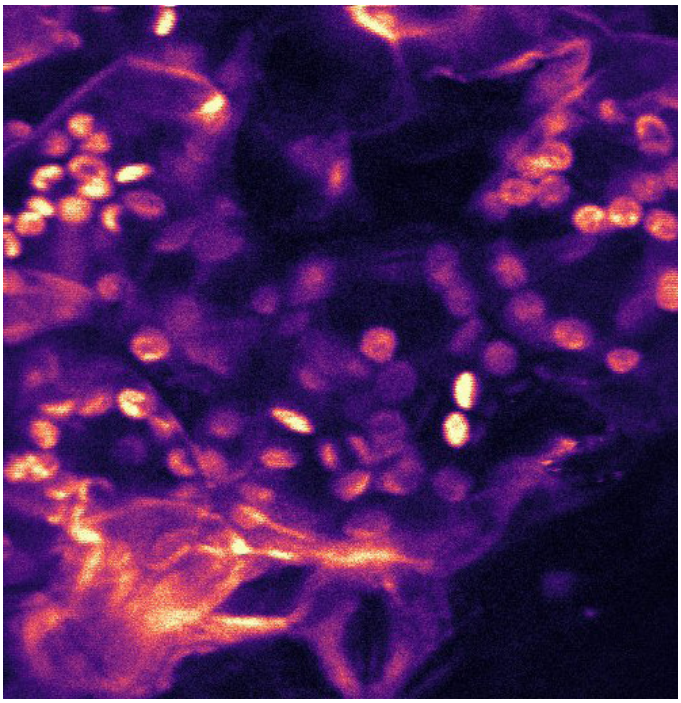
In other fluorescence techniques the laser will either excite the fluorescent dyes throughout a sample or, through a cone of light down to the focal plane (e.g. in confocal microscopy). This leads to more out of focus excitation, causing faster photobleaching (the gradual decline of most fluorophore's ability to fluoresce) and phototoxicity (the toxic effect of activated fluorophores on cells).

An additional benefit occurs through the use of infrared wavelengths. Light from the visible spectrum scatters a lot in biological tissue; severely limiting the depths it can penetrate with enough power to excite a fluorophore. The infrared lasers used for two-photon microscopy scatter much less. This means infrared laser light has enough power to excite fluorophores up to around 1 mm in living tissues. In comparison, single photon confocal microscopy can only penetrate to about 200 µm.

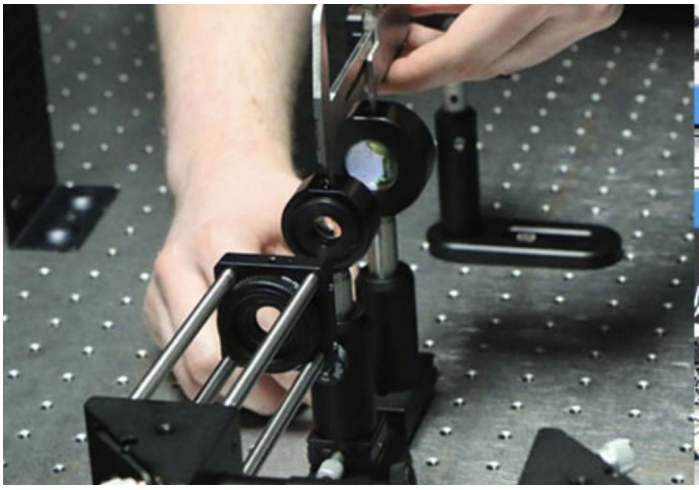
The first commercially available two-photon microscopes were introduced in 1996. More than 20 years later they have increased our understanding in numerous areas of biology. Now hundreds of labs around the world have adopted two-photon microscopy to overcome the limitations of alternative fluorescence microscopy techniques.

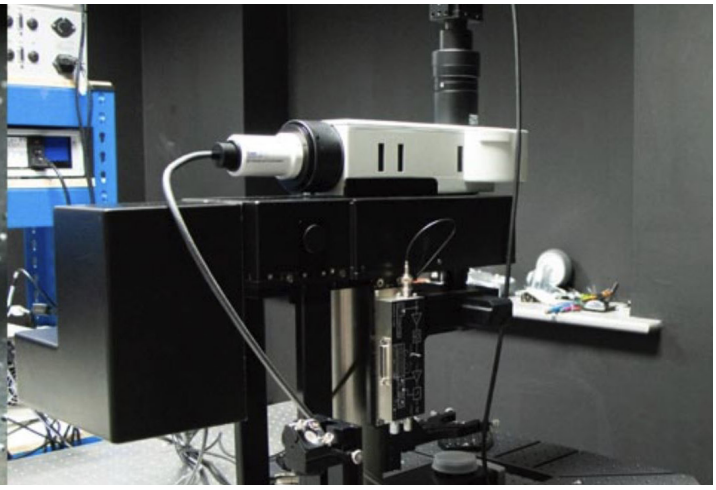


The underside of an American Oak leaf imaged using two-photon excitation on the HyperScope multiphoton imaging system. This shows a single focus plane taken from a 3D projection of the entire imaged volume.



Autofluorescent plant leaf imaged using two-photon galvo imaging on the HyperScope RGG multiphoton system.





How to align your laser for two-photon imaging

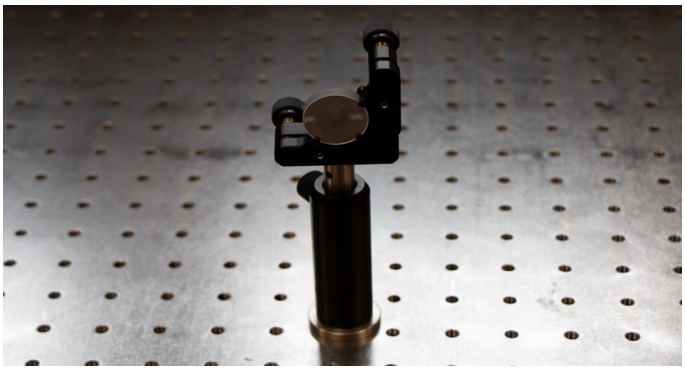
Two-photon microscopy uses a laser to excite fluorescent molecules (fluorophores) within a sample through emitting short pulses of light at high power. This step-by-step guide demonstrates how to safely align a laser for two photon microscopy.

Safety advice

Anybody working with lasers should be fully trained and follow the applicable institutional and national laser safety guidelines and legislation. These safety tips are for guidance only and can no way replace laser safety training.

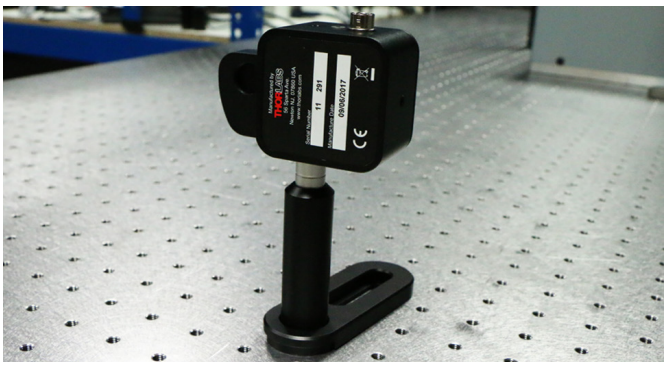
- Do not enter laser rooms unless trained or accompanied by someone who is trained and authorised
- Do not look into the laser beam
- Wear suitable laser safety eye-wear
- Keep room lights on brightly if possible
- Locate and terminate all stray laser beams at an absorbing or diffusely reflecting surface
- Avoid stray laser beam reflections as they could be hazardous to the eyes and skin
- Remove all personal jewellery, including watches
- Clamp all optical components
- Be cautious using IR Detector Cards with glossy surfaces
- Do not use reflective tools
- Keep beams horizontal, if this isn't possible, be very careful with non-horizontal beams (e.g. flipping mirrors and periscopes)
- Ensure beams are well above or below eye level
- If you need to bend down, always look away from the table
- Do not bend below beam height
- Be extremely cautious when leaning down to beam-level
- Think twice before leaning to table level to get a better look at your experiment
- Do not forget non-optical hazards (e.g. tripping, fire risk)
- Encase the beam as much as possible using beam tubing
- Shutter the beam when putting optical elements, such as mirrors, in place
- Use a beam block behind each optical component to ensure there are no stray laser beams while setting up the next element

The equipment



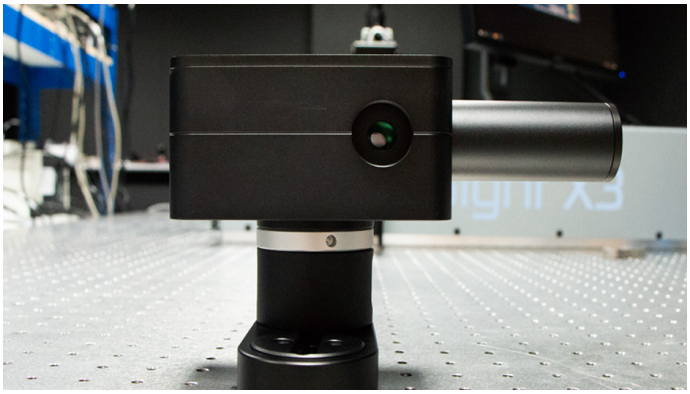
Steering mirrors

Used for gross beam steering and fine directional tuning.



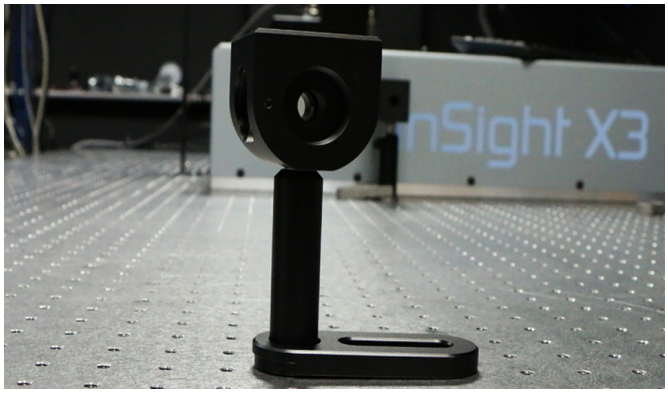
PICK mirror

Samples the beam to inform you of the power of it.



Manual Intensity Controller

Changes the intensity of the beam. Attenuates the beam before it enters a Pockels cell to control the maximum energy through the system. Only needed if the available laser power exceeds Pockels cell input level.

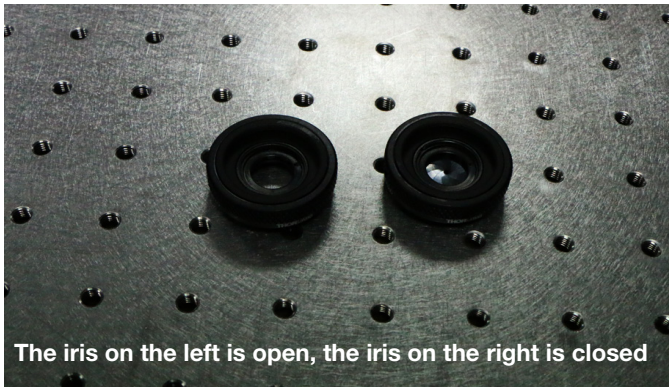


Shutter

Blocks the beam when it isn't being used. The shutter only opens immediately prior to image acquisition to protect the sample from the laser beam and the user from exposure to stray laser beams.

Power meter

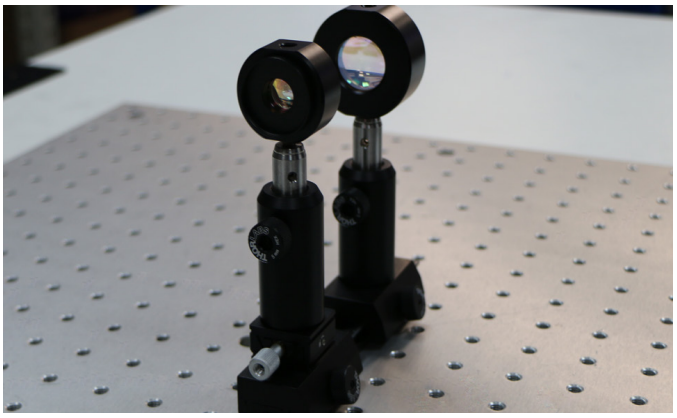
Used to measure the beam power.



The iris on the left is open, the iris on the right is closed

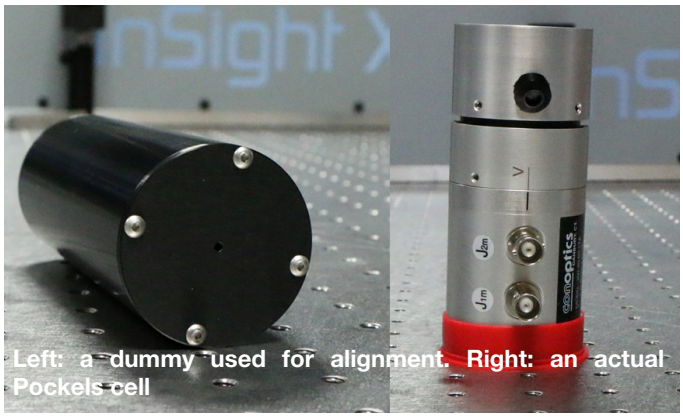
Irises

The laser is aligned to the centre of the irises.



Beam expander

The beam expander increases the diameter of the laser beam in order to properly overfill large back aperture objectives.



Left: a dummy used for alignment. Right: an actual Pockels cell

Pockels cell

An electro-optical modulator that attenuates and controls the beam when speed is essential, for example in photostimulation experiments. Faster and more precise than using the half-wave plate/polarising

Aliging the laser

1. Plan your light path, depending on the amount of space you have available. Where will you place mirrors and Pockels cells? Where will your attenuation be? It is best to have at least two mirrors in front of Pockels cells and beam expanders to help with alignment.

2. Position mirrors, Pockels cells and any other equipment where you have planned it to be.

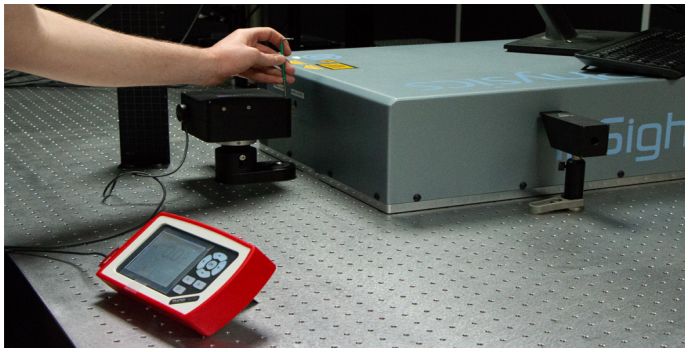
3. Put the laser into alignment mode. The alignment mode should never be used for more than 20 minutes, followed by at least a 10 minute break to allow the laser to cool off.

Please note, the alignment mode of the laser does not protect eyes; it is designed to protect equipment

4. Attenuate the laser by lining up the manual intensity controller with the laser output. Once this has been aligned, open the Manual Intensity Controller lid and rotate the half waveplate to attenuate the beam.

We attenuated the beam from 200mW to 30mW using the manual intensity controller and power meter.

Here, the beam is being aligned through the centre of the Manual Intensity Controller.



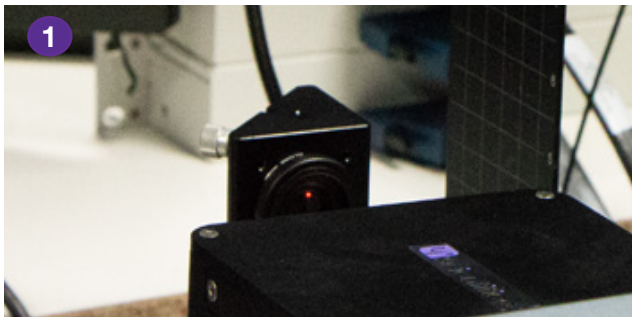
The half waveplate within the Manual Intensity Controller is being used to attenuate the laser beam.

5. Shutter the laser to avoid injury.

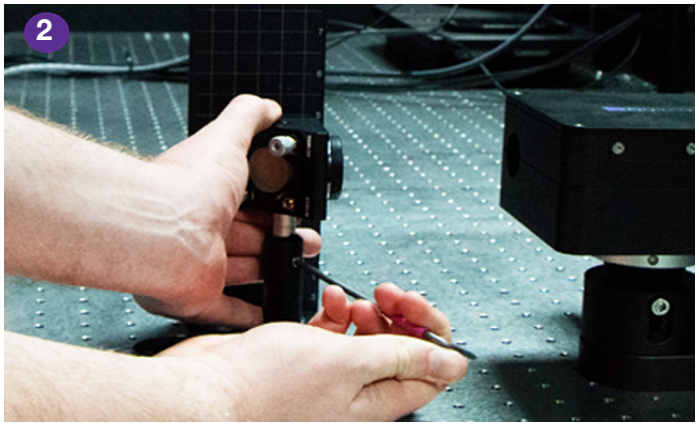
6. Attach irises to the front of the mirrors. The laser will be aligned to the centre of the irises.

7. Unshutter the laser.

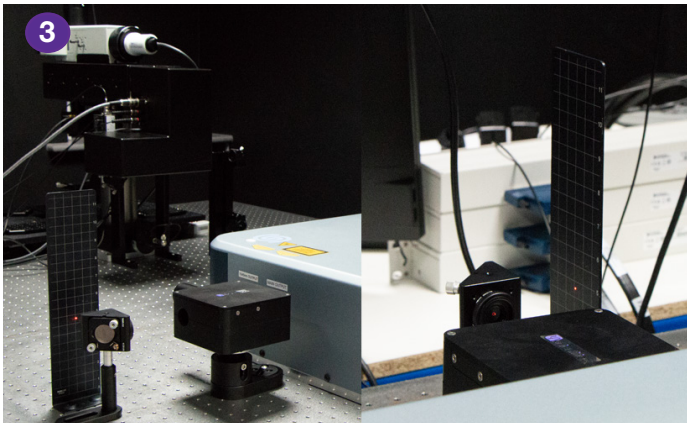
8. Set the height of the first mirror – the beam needs to hit the centre of the iris. Use a set screw to loosen and adjust the height of the mirror accordingly. Use a beam block to stop stray beams - see images 1, 2 and 3 for guidance.



The mirror height has not been adjusted, so the laser beam is not aligned to the centre of the iris.



The mirror height being adjusted, using a set screw.

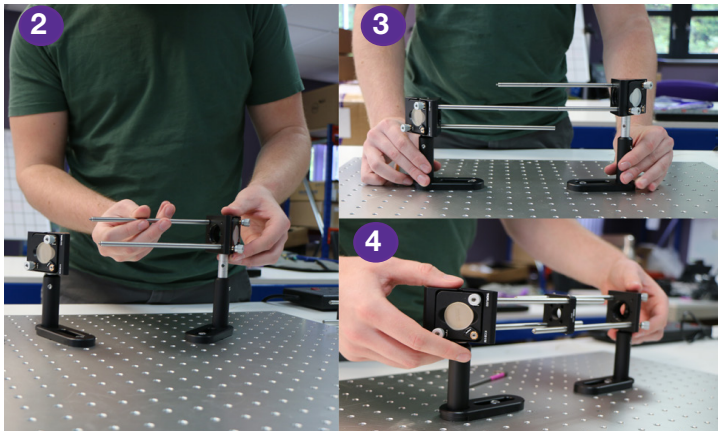


The mirror is now the correct height and the laser beam is aligned to the centre of the iris. The beam block is blocking the beam that is reflected by the steering mirror.

9. Use this first mirror to set the height of the other mirrors – this can be done in two ways:

a) Using a cage plate, insert rods into opposite holes on the two mirrors and then join the two mirrors together to ensure they are the same height. This method is the most accurate. See steps 1 - 5 below.

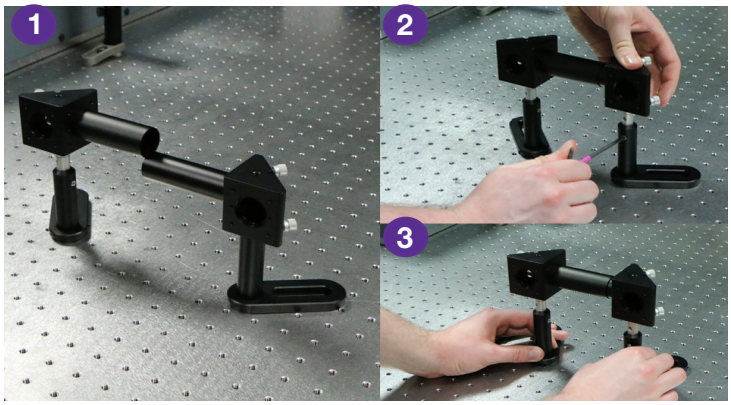
The first mirror is a different height to the other mirrors - this will be used as a template to set the other mirrors to the correct height.



A cage plate and rods are being used to align the mirrors and ensure they are the same height.

The two mirrors are now the same height.

b) Screw a beam tube into the two mirrors then align them – the tubes should be able to slide in and out with as little resistance as possible. Refer to steps 1 - 3 below.

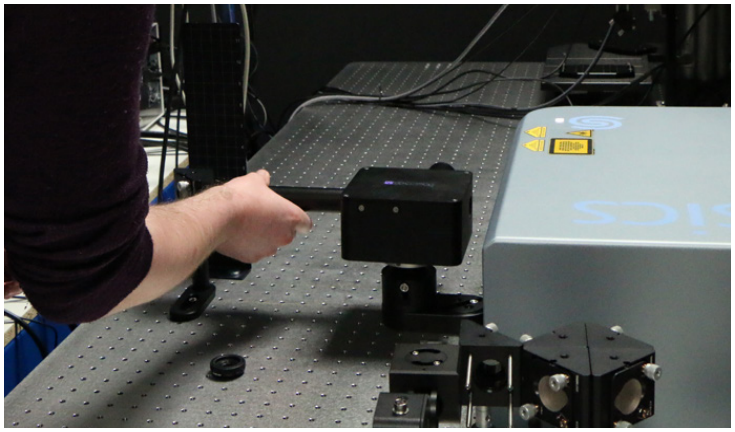


Instead of using rods and a cage plate, the mirrors are being aligned by inserting tubes & lining them up.

10. Put the mirrors in place, again ensuring they are correctly aligned so the laser spot is in the centre of the iris.

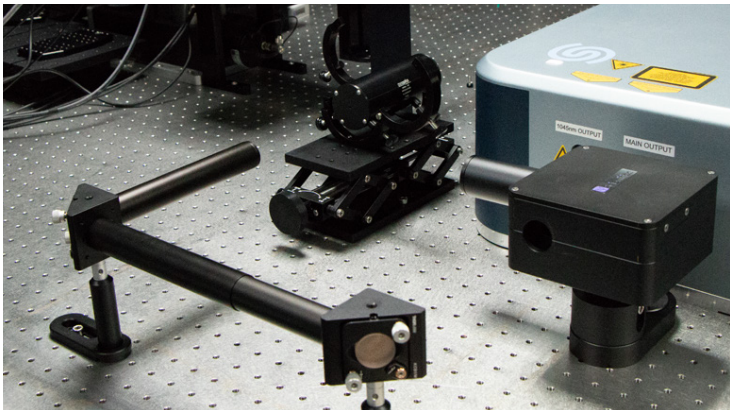
Left: laser beam is mis-aligned as it is not in the centre of the iris.
Right: laser beam is aligned to the centre of the iris.

To prevent injury from the laser beam and stray laser beams: shutter the laser before positioning each mirror, add a beam block behind each mirror and use beam tubing.



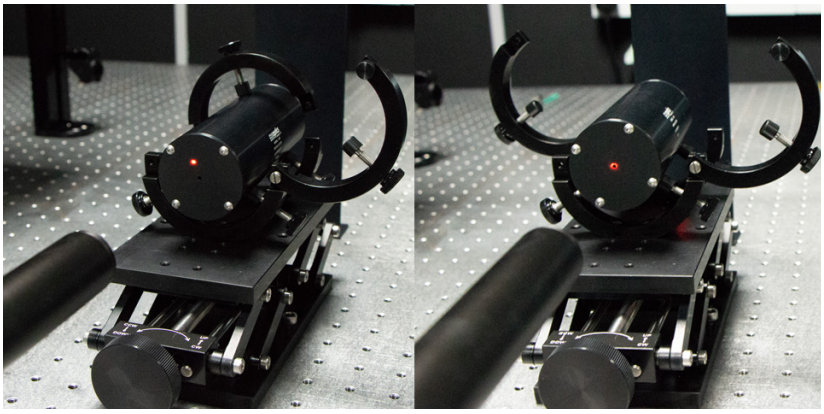
Beam tubing is being added to encase the laser beam between the manual intensity controller and mirror.

11. Using two mirrors enables you to alter the direction as well as parallel shift the laser beam, making it easier to align the beam expander and Pockels cell. If the beam is not aligned, it will not go through the Pockels cell.



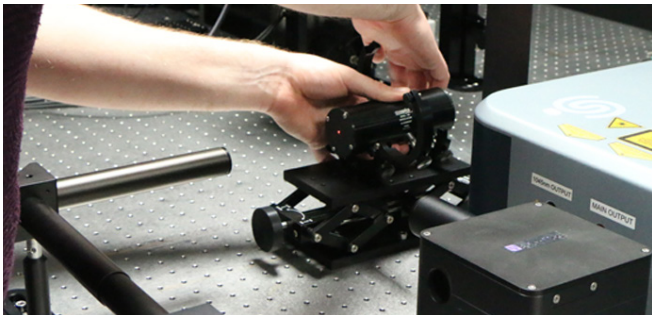
Two mirrors are directing the laser beam to the dummy Pockels cell.

12. Optimise the alignment for the Pockels cell. Walk the beam* to ensure it travels through the centre of the Pockels cell. Using two mirrors, make sure the light is turning at a 90 degree angle. Ensure a beam dump is used to block the rejected beam from the top of the Pockels cell (this is not shown in the image below as a dummy is being used).

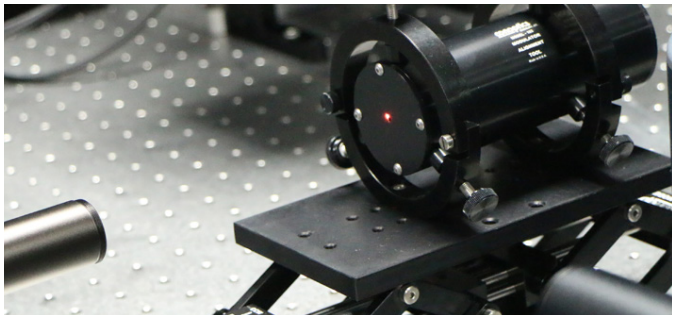


Left: the laser beam is not aligned to the centre of the Pockels cell.
Right: the laser beam is aligned to the centre of the Pockels cell.

13. Close the Pockels cell holder using the screws on the side, to make sure it stays in place. Once you have done this, fine-tune the Pockels cell alignment.



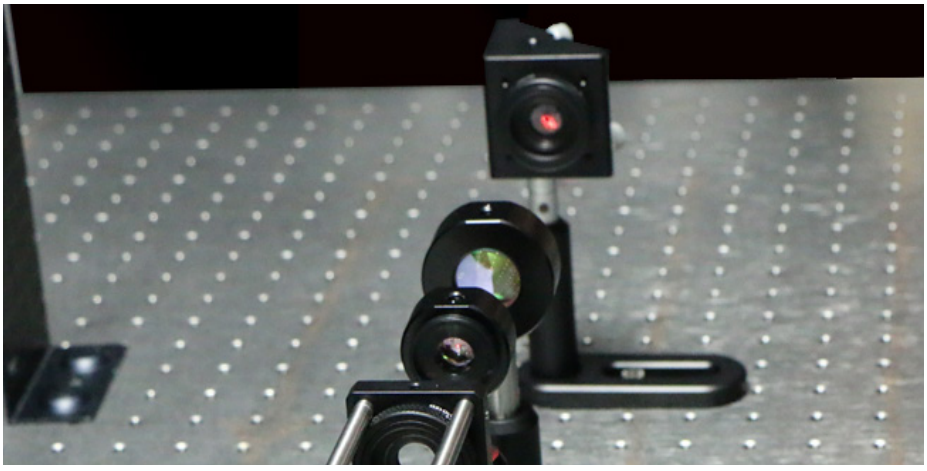
Using the screws on the side of the Pockels cell holder, the holder is being closed in order to secure the Pockels cell.



The Pockels cell is secure in the holder and the laser beam is aligned to the centre of the Pockels cell.

14. Optimise the alignment for the beam expander. Walk the beam* to ensure it travels through the centre of the beam expander. Use two irises, placed either side of where the beam expander will be positioned, for guidance. If the beam is misaligned through the beam expander, it will exaggerate the direction of the beam.

*See next section for how to do this.



The laser is aligned to the centre of the beam expander.

15. The beam expander expands and collimates the beam, so when you move the beam block, the size of the beam should remain the same. If it gets bigger or smaller, the distance between the two lenses is incorrect.

16. Install the final mirrors, you will use this to align the beam to the periscope, which couples the light into the microscope.

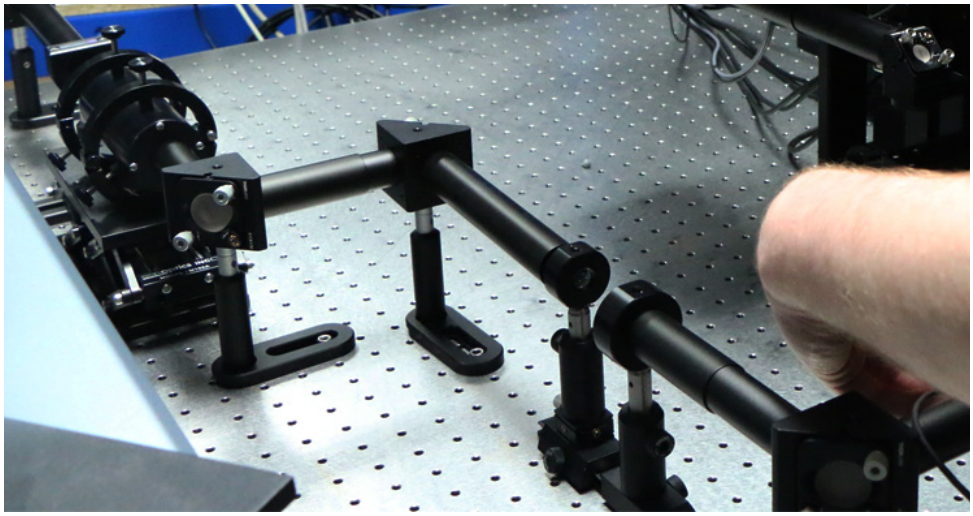
17. Install the shutter and pick mirror. These can be positioned anywhere, as long as they are positioned after the Pockels cell. Mirror realignment will be needed now, as the pick mirror parallel shifts the beam.

18. Ensure the mirror and bottom of the periscope are at the same height.

19. Align the periscope – this can be done in multiple ways. To avoid damaging your eyes, we recommend using a camera or camera phone (on selfie mode) to look underneath the periscope, to see if the beam is aligned to the centre of the top periscope mirror.

20. Align from the top of the periscope into the microscope.

21. Insert tubing to encase the laser beam as much as possible, to reduce the risk of injury.

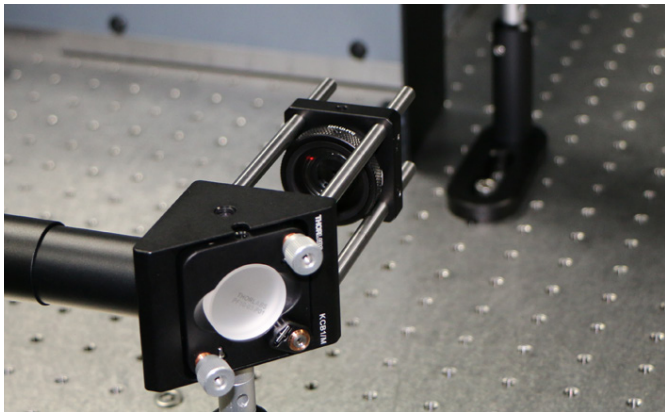


Tubing is being inserted as much as possible to encase the laser beam.

*Walking the beam

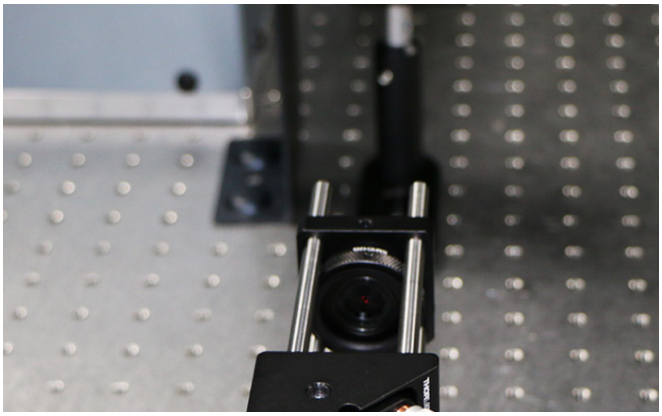
We have referred to walking the beam twice in this article. This involves using two steering mirrors to align the beam to the centre of two irises. Here are detailed steps of how to do this:

1. Using the levers on the first steering mirror (not pictured), align the beam to the centre of the first iris.



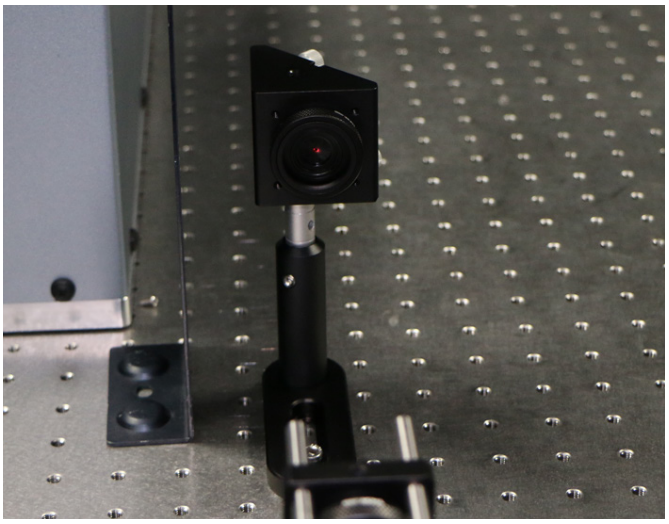
Here, the laser beam is not aligned to the centre of the first iris. Using the white levers on the first steering mirror (not pictured), the beam can be aligned to the centre of this iris.

2. Open the first iris so that the beam hits the second iris.



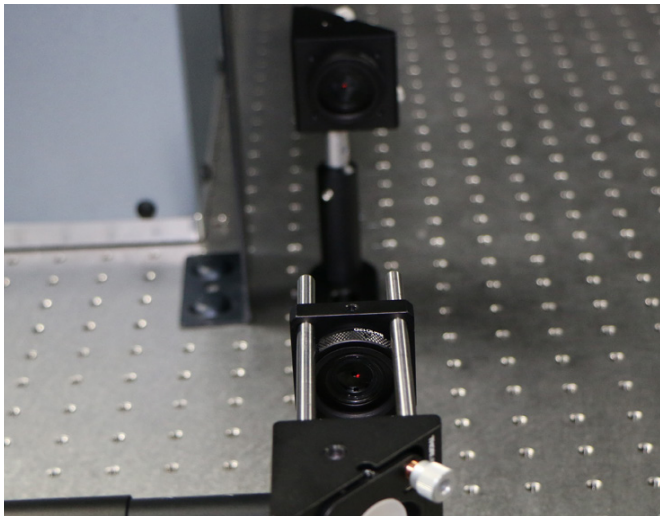
The first iris is partially open so the laser beam travels to the second iris. The laser beam is aligned to the centre of the first iris, but is not aligned to the centre of the second iris.

3. Using the levers on the second steering mirror, align the beam to the centre of the second iris.



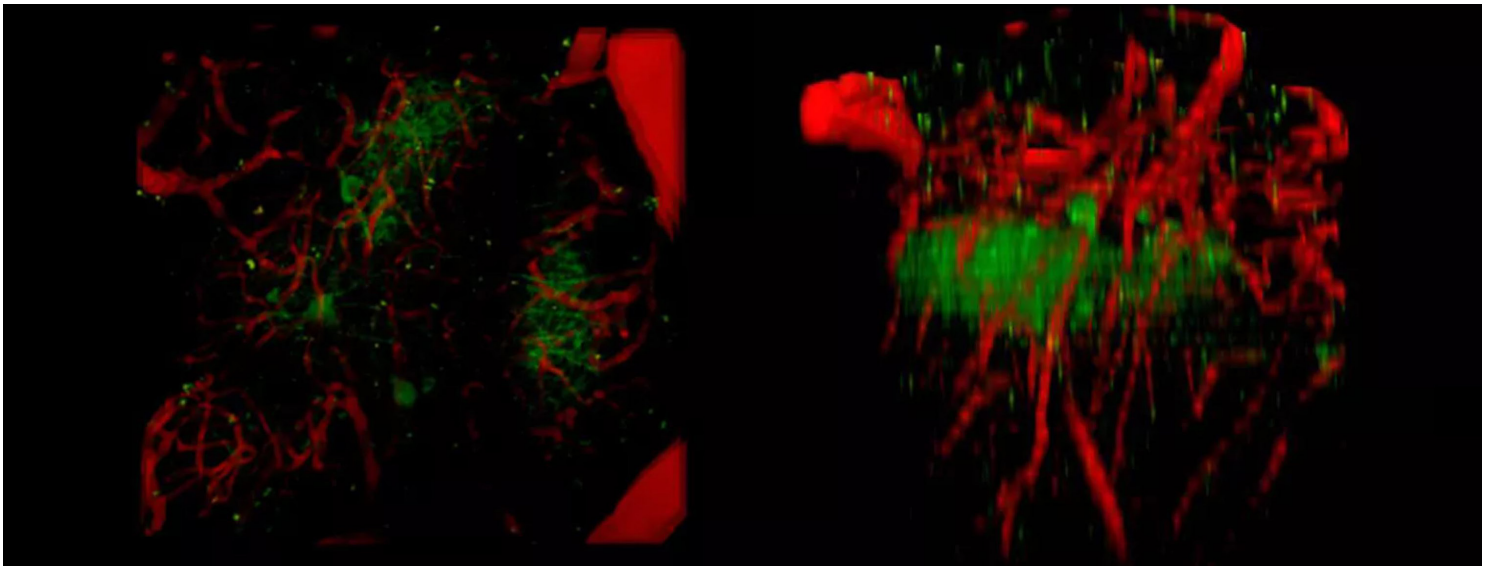
Using the white levers on the second steering mirror, pictured above, the laser beam has been aligned to the centre of the second iris.

4. Close the first iris again, and re-align the beam to the centre of this iris.



The beam is now aligned to the centre of the second iris, but not to the centre of the first iris. This needs to be re-aligned.

5. Repeat these steps until the laser beam is aligned to the centre of both irises.



Three-photon imaging: How it works

Three-photon imaging is a fluorescence microscopy technique that enables deeper imaging compared to two-photon or one-photon fluorescence microscopy.

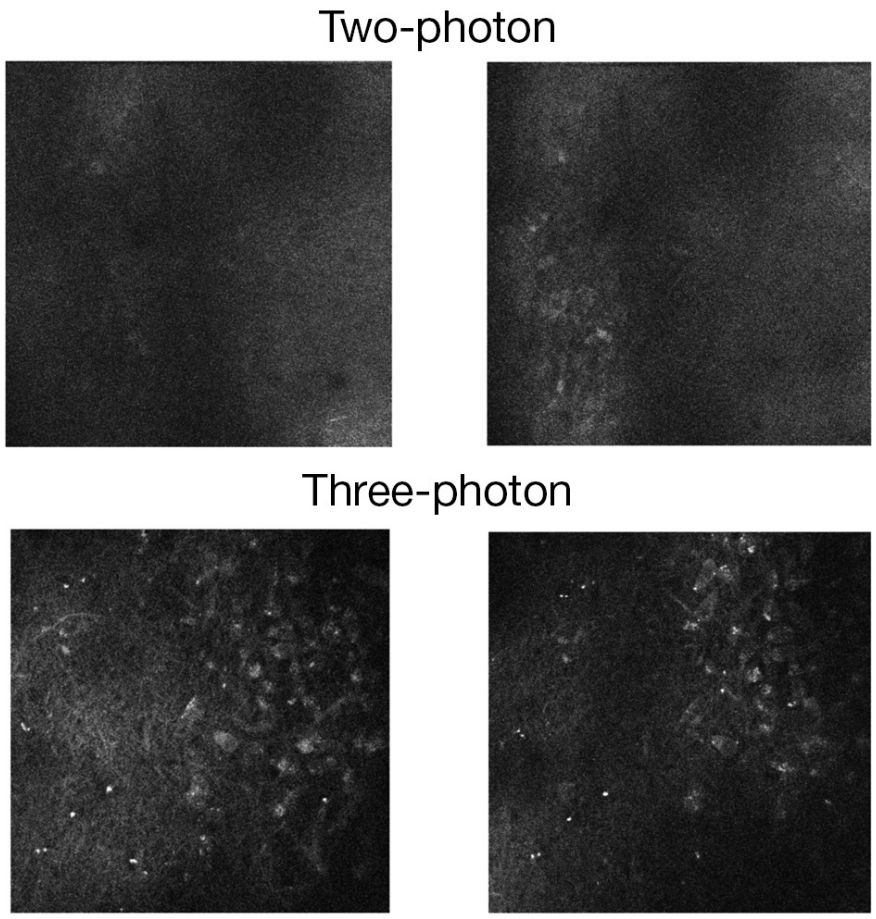
Similar to two-photon imaging, where two photons simultaneously interact with a fluorescent molecule, in three-photon imaging, three-photons simultaneously interact with a fluorescent molecule to excite it to a higher electronic state.

The three photons that excite a fluorophore will have longer wavelengths and lower energy than those used to excite the same fluorophore in one- or two-photon fluorescence imaging, for example excitation of green fluorescent protein (GFP) requires:

1 photon with a wavelength of 480nm
2 photons each with a wavelength of 910nm
3 photons each with a wavelength of 1300nm

Advantages

Three-photon imaging is ideal for imaging deeper in scattering tissue, or when imaging through thin scattering layers. Due to the light having a longer wavelength, it is able to penetrate deeper into tissue. The light scatters less, enabling clearer images of structures deep in scattering tissue to be obtained. Fluorophores deeper in tissue can be excited, and, as with other optical sectioning techniques, structures can be visualised in 3D.



Mitral cells of the mouse olfactory bulb. Left is with dura and right is without. Top: taken using two-photon imaging. Bottom: imaged with three photons, the three-photon images have a higher contrast and show finer details.

How three-photon imaging works

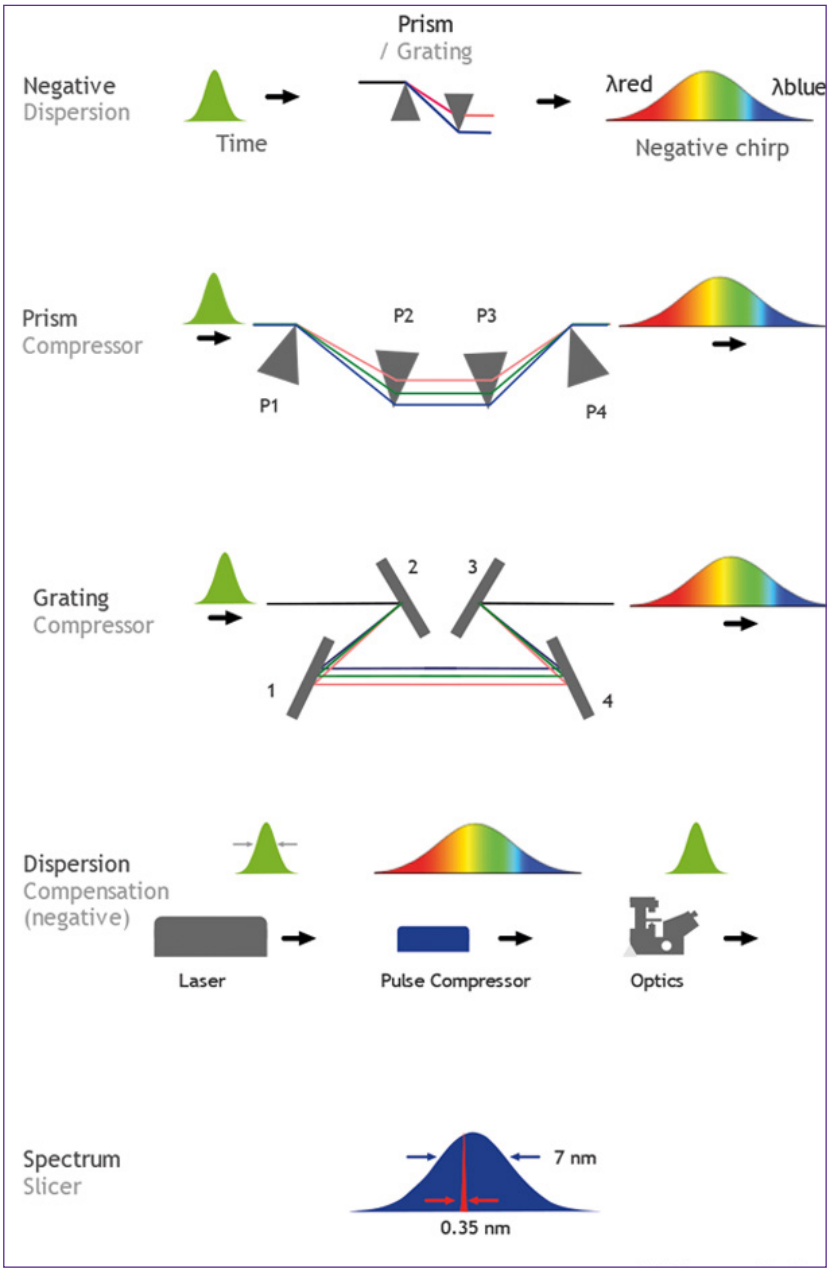
To achieve three-photon excitation, the three photons need to interact with the fluorophore simultaneously (i.e. within less than 1 femtosecond of each other). Therefore, light with a high photon density needs to be used. This is achieved by using fast laser pulses that have high peak power.

A special high-powered laser source is required to achieve the higher photon density and longer wavelengths required for excitation by three photons. The commonly used approach is based on a high power fibre laser pumping an optical parametric amplifier (OPA), which takes light of one wavelength and turns it into light of two different wavelengths. One of these beams has a shorter wavelength than the initial pumped laser. This is the signal beam and can be used for two-photon imaging. The second beam has a longer wavelength than the initial pumped laser. This is the idler beam and is ideal for three-photon imaging.

The idler beam is passed through a pre-chirper (pulse compressor), to ensure the shortest possible pulses at the sample.

The pre-chirper achieves the shortest possible pulses at the sample by introducing a negative chirp into the idler beam. Laser pulses are stretched by dispersion of optical material when they pass through optical components such as lenses. This is because the different wavelengths that make up the laser light travel at different speeds through the optical material. Introducing a negative chirp counteracts this effect, so when the laser pulse goes

through glass, the different wavelengths leave the glass at the same time. This is known as dispersion compensation and creates a short pulse with a high photon density.



How an OPA achieves dispersion compensation to ensure pulsed light with a high photon density hits the sample. Image courtesy of APE

Limitations

Due to a higher density of photons, and therefore more light being used in three-photon imaging, photodamage and photobleaching are more likely to occur compared to two-photon imaging. Care needs to be taken to keep excitation powers at a biocompatible level.

The main limitation of three-photon imaging is that there is no ability for resonant imaging, due to the very low repetition rate of the laser.

For more information about any of the imaging techniques described in this magazine, please contact our team of experts at info@scientifica.uk.com or visit www.scientifica.uk.com

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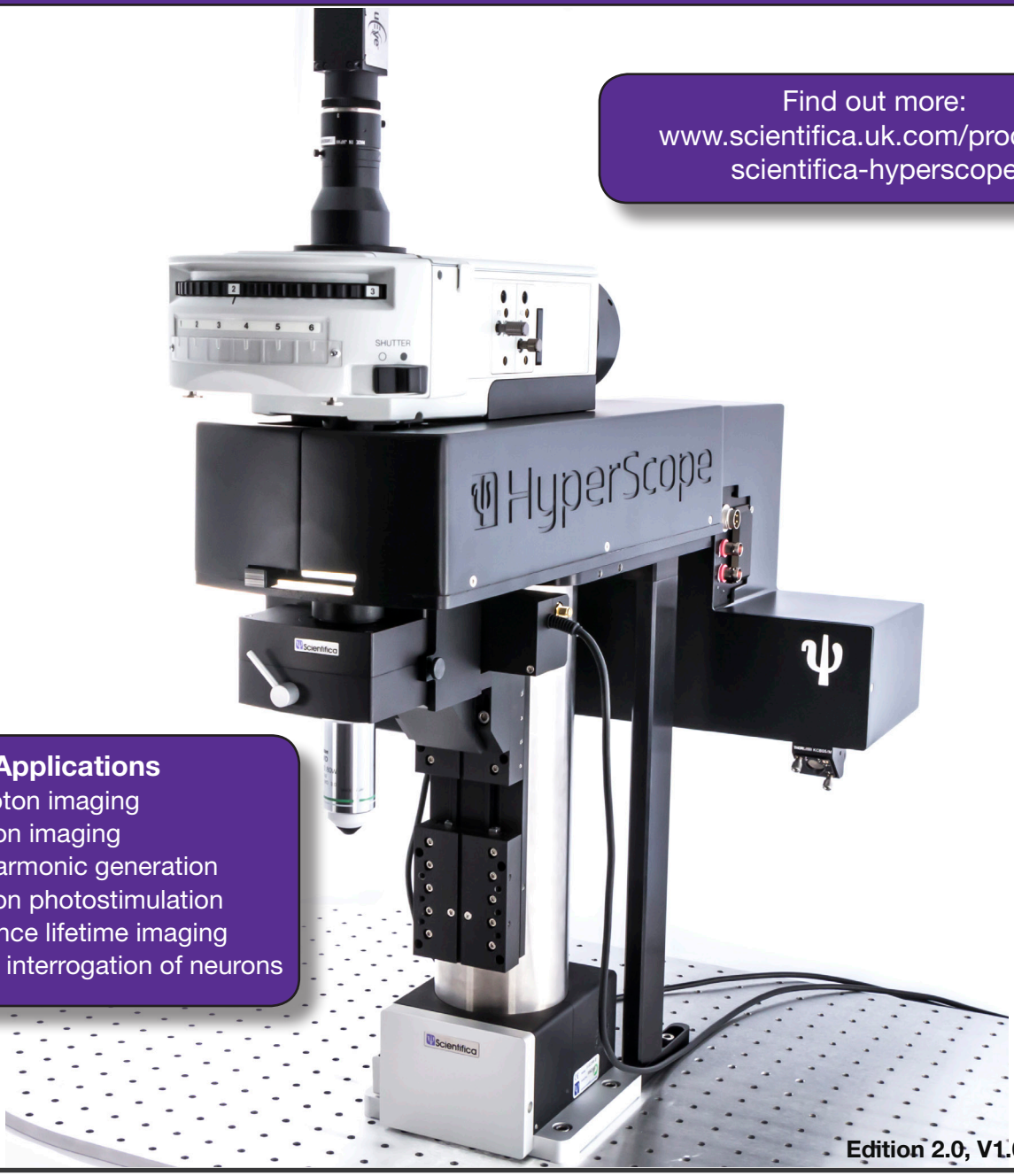

Applications
- Three-photon imaging
- Two-photon imaging
- Second harmonic generation
- Two-photon photostimulation
- Fluorescence lifetime imaging
- All-optical interrogation of neurons

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