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THORLABS

MPM-SCAN4 - January 9, 2017

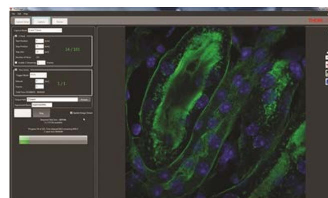
Item # MPM-SCAN4 was discontinued on January 9, 2017. For informational purposes, this is a copy of the website content at that time and is valid only for the stated product.

Hide Overview

OVERVIEW

Features

- For Customers Building Their Own Multiphoton Microscopes
- Acquire Images at up to 400 Frames per Second
- Compact, Versatile Design Accommodates Large Specimens and Apparatuses
- Two Ultrasensitive PMTs in Non-Descanned Geometry
- Full Support for Two Image Acquisition Suites
  - ThorImageLS from Thorlabs (See *Software* Tab)
  - ScanImage 4.x and 5.x
- Includes Galvo-Resonant Scan Head, Scan and Tube Lenses, Two PMTs, Computer, and Electronic Control Unit


Click to Enlarge
ThorImageLS Capture Tab (See *Software* Tab for More Details)

Thorlabs' Multiphoton Essentials Kit helps customers build their own multiphoton microscopes. Consisting of a galvo-resonant scan head, NIR scan and tube lens combination, and two non-descanned PMTs in a single, preassembled enclosure, it incorporates many of the same technologies and components used by our Bergamo II Series Multiphoton Microscopes.

Our internally developed resonant scanner offers image scan rates up to 400 frames per second (512 x 32 pixels; see *Specs* tab for details). Once a femtosecond laser and suitable objective are added, the resulting system is ready to perform multiphoton experiments with two-channel detection.

ThorImageLS™
This kit includes ThorImageLS, a software suite for acquiring images and video, and is fully supported by ScanImage 4.x and 5.x. ThorImageLS was developed side by side with our multiphoton microscopy platforms and is seamlessly integrated with our resonant scanner. It offers a workflow-oriented interface that guides you step by step through common scan types like Z series for volumetric scans, time series for dynamic scans, and image streaming, and saves the data directly as a lossless TIFF that is viewable in any image analysis program, like ImageJ. It can also export image streams as animations in AVI format. For more information, please see the *Software* tab.

Thorlabs recognizes that each imaging application has unique requirements.
If you have any feedback, questions, or need a quotation, please contact
ImagingSales@thorlabs.com or call (703) 651-1700.

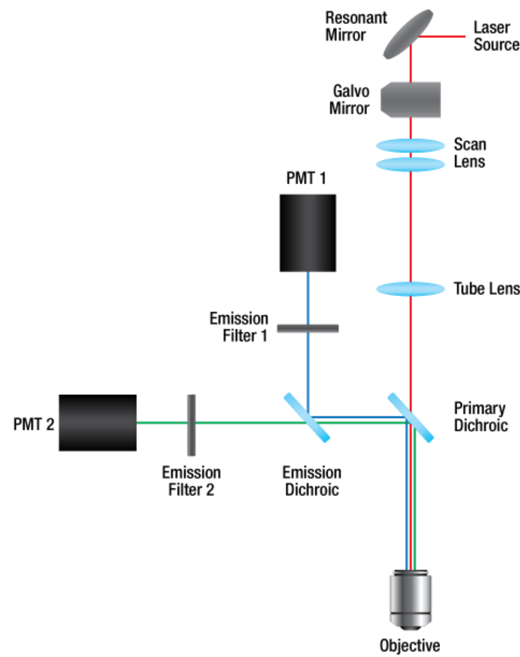
Hide Technology

TECHNOLOGY

Superb Performance

We started from a clean sheet of paper to design our multiphoton scanning and detection optics, allowing us to specifically optimize for laser scanning with a near-infrared beam and maximal collection of the two-photon fluorescence signal.

Non-



Optical Path of Multiphoton Essentials Kit

Descanned PMT Detectors

Deriving more signal from fewer photons is the fundamental goal of any detection system. To this end, we employ ultrasensitive GaAsP PMTs. By positioning the PMTs immediately after the objective (a "non-descanned" geometry), light that is scattered by the sample still strikes the PMTs and adds to the collected signal. This is a unique benefit of multiphoton microscopy. Keeping the length of the optical path to a minimum greatly enhances overall detection efficiency when imaging deep in tissue.

NIR Optical Excitation Path

The Multiphoton Essentials Kit supports excitation wavelengths extending into the NIR (680 - 1400 nm), making a wide range of fluorophores accessible and allowing others to be excited more efficiently. This wavelength flexibility is key to taking full advantage of the ever-evolving spectrum of fluorescent proteins and maximizing imaging depth and resolution.

Galvo-Resonant Scanner

Whether visualizing Ca^{2+} influx, microcirculatory blood flow, or limiting smearing from motion, temporal image resolution is essential. With a maximum scan rate of 400 frames per second (512 x 32 pixels), some of the fastest physiological changes become observable. Thanks to optimized data acquisition algorithms, our resonant scanners can also achieve high resolution, with a maximum pixel density of 4096 x 4096. By adding our high-speed laser modulator (part of our Multiphoton Beam Conditioner), dynamic, targeted path scanning of up to 400 regions per second is achievable.

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ImagingSales@thorlabs.com or call (703) 651-1700.

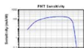
Hide Specs

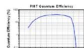
SPECS

Click to Enlarge
Optical Path of Multiphoton Essentials Kit

Multiphoton Essentials Kit	
Excitation	
Wavelength Range	680 - 1400 nm

Objective Pupil Diameter	20 mm (Max)
Field of View	16 mm Diagonal Square (Max) at the Intermediate Image Plane 700 µm x 700 µm at Sample (with Nikon 16X Objective)
Microscope Objective Thread	M32 x 0.75 (Adapter for M25 x 0.75 Objectives Included)
Scanner	X: 8 kHz Resonant Scanner Y: Galvonometric Scan Mirror
Scan Speed	30 FPS at 512 x 512 Pixels 400 FPS at 512 x 32 Pixels 2 FPS at 4096 x 4096 Pixels
Scan Mode	Line, Square, or Rectangle
Scan Zoom	1X to 36X
Scan Resolution	Up to 2048 x 2048 Pixels (Bi-Directional) Up to 4096 x 4096 Pixels (Unidirectional)
Primary Dichroic	Reflective Below 680 nm; Transmissive from 680 - 1400 nm
Detection	
Backward Direction	Two Ultrasensitive GaAsP PMTs
Collection Optics	Full Field of View User-Changeable Emission Filters and Dichroic Mirror
Wavelength Range	300 - 720 nm


Click to Enlarge


Click to Enlarge

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Hide Software

SOFTWARE

ThorImageLS: Intuitive Workflow-Oriented Software Suite

ThorImageLS™ was developed side-by-side with our multiphoton microscopy platforms to ensure seamless, logical, and intuitive integration between software and hardware. Our workflow-oriented interface only displays the parameters you need for each scan series (such as Z series for volumetric scans, time series for imaging of dynamics, or bleaching series for photoactivation/uncaging experiments). Each software mode offers a gentle learning curve, guides the researcher step by step through data acquisition, and will have you capturing images with just a few clicks.

We are pleased to announce that ThorImageLS' full source code is available. E-mail us for your copy.

A complete solution for our microscopy platforms, ThorImageLS provides control of not only the microscope but also a wide range of accessories. In-line panels control the position of our motorized XY and Z stages, the laser power at the sample, and the wavelength of tunable Ti:Sapphire lasers for multiphoton microscopy. This high degree of automation minimizes distractions, allowing you to keep your focus on your research.

Download

Version 3.0 - November 2, 2016

Contact Us

Please contact ImagingTechSupport@thorlabs.com to obtain the latest ThorImageLS version compatible with your microscope. Because ThorImageLS 3.0 adds significant new features over 2.x and 1.x versions, it may not be compatible with older microscopes. We continue to support older software versions for customers with older hardware.

New Features

- Added support for DDR05(/M) fast power control device, which allows for faster power ramping acquisitions
- Added support for the entry level Galvo-Galvo Confocal system (no separate digital acquisition board necessary)
- Added fine two-way calibration, which provides fewer two-way adjustments when changing field sizes
- Added ability to save Galvo-Galvo offset and scale values to ThorConfocalGalvoSettings.xml file
- Added option to save .tiff files compressed or uncompressed
- Added option to save only enabled channels as raw image files
- Added ability to save snapshot image either as a single image or as an experiment
- Added ability for multi-location imaging on platforms with supporting stages (Bergamo, MCM3000 and High-Speed Motorized XY Scanning Stages)
- Includes new UI for defining and navigating multi-location

User Interface (UI) Improvements

- Changed the layout for the hardware setup window
- Added mouse scrolling functionality in settings editor
- Removed un-necessary .xml files in settings editor view
- Moved center scanners and resonance scanner from always visible to the area control advanced panel in Capture Setup
- Added second column option for capture setup display
- Moved field size entry to under scan area cartoon
- Added dropdown list for most popular pixel density settings.
- Added +/- buttons for Galvo-Galvo angle control
- Changed the Z slider bar to objective graphical
- Replaced coarse/fine buttons with buttons labeled increase/decrease
- Changed Z units from mm to µm
- Added visibility option for "Set Zero" feature in XY and Z panels
- Added visibility for invert option in Z control panel
- Added visibility option individual light path controls

regions
- Added ability to invert scanners (flip horizontal and vertical) for Galvo-Resonant and Galvo-Galvo systems
- Added ability to invert stage directions for Bergamo, MCM3000 and High-Speed Motorized XY Scanning Stages
- Added ability to use an ROI mask (ROIMask.raw) as a Pockels mask. This includes updating the mask in the UI
- Added ability to use Pockels power ramping during fast Z acquisitions
- Added high temporal resolution image capture spacing. Allows the user to set a delay between frame acquisitions
- Added ability to sequentially capture images using different hardware settings, such as Channel, PMT, Laser and Power
- Added ability for High-Speed Motorized XY Scanning Stages laser calibration to linearize power output
- Added support for a secondary Z panel
- Added offset control support for PMT1000 and PMT2100 devices
- Added ability to image and bleach simultaneously
- Added option to save bleach images in Raw image format
- Added ability to turn off the computer monitor when starting experiment capture
- Added ability to synchronize the Start and Stop of ThorSync with ThorImage Capture start and stop
- Added orthogonal view functionality for Z stack acquisitions in Image Review
- Added new script commands to move X, Y and Z motors between script acquisitions
- Added support for Tiberius two-photon laser

Fixed Bugs

- Fixed bleaching capture fields from being editable during capture
- Fixed raw image review not supporting 3D display
- Fixed PMT3 and PMT4 voltage range setting saved in the template not being used when running a Script
- Fixed galvo-galvo line scan setting the Y scale incorrectly when changing the X scale
- Fixed error when galvo-galvo snapshot appears to lock up with large pixel density setting and dwell time, and added abort button
- Fixed standard sensitivity PMTs not being set to 0 when the display indicates 0 after a hardware reset
- A 4 channel snap-shot image is now saved as a multi-page image instead of RGB
- Added an adjustable phase shift parameter to correct the pockels waveform sometimes being out of phase across the image
- Removed un-necessary files to correct for the application appearing locked up when selecting certain .xml files in the settings editor takes a long time

Renamed the coherent control panel to multiphoton laser control
- Display summary and status for collapsed panels in Capture Setup
- Added features to histogram control:
  - Black and white point fields
  - Connector between black and white point fields to help locate mid-point
  - Ability to enlarge single or all histograms
  - Log scale display option
- Stats chart and stats window changes:
  - Change how they are displayed. Selecting to close the window now de-selects visibility
  - Added ability to save a chart as a .jpg
  - Added ability to save table data as .csv, .txt or .raw
  - Changed the chart Y scale to scientific notation
  - Set the chart X axis limit to the range of data
- Added display option for line profile window
- Color settings changes:
  - Added more look up table (LUT) colors: BlueStat, CyanHot, GrayStat, GreenStat and RedStat
  - Allow the same LUT for multiple colors
  - Enhanced display of min and max when viewing the gray scale of single image features
- Eliminated zoom-level edit dialog and replaced with user-entered zoom field
- Changed experiment naming by adding a separate iteration field
- Added dialog to suppress the "File Name Exists" prompt
- Changed browsers to a more useful interface
- Removed the intermediate menu when selecting image review
- Added the ability to save more experiment information, such as Galvo-Galvo angle value and pinhole size
- Z stack experiments open Z slider to mid-range
- Added Z and T unit display for Z and time index
- Image review play is now in a continuous loop until manually stopped
- Changed Galvo-Galvo pixel dwell time scale bar to single bar

Experimental Techniques

- Ramp Power with Sample Depth to Minimize Damage While Maximizing Deep Signal-to-Noise

Equipment Control

- Control Power of Independent Excitation Lasers
- Insert/Remove Dichroic Mirrors for Different

- Customize Acquisition Parameters for High-Speed Z-Stacks or Image Streams
- Select Region of Interest to Photoactivate/Bleach with an Easy-to-Use Interface

Scan Types

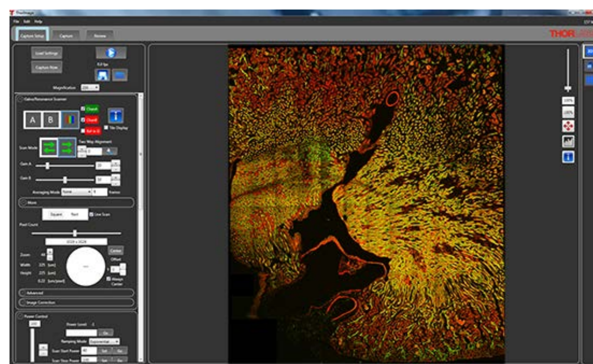
- Integrate with Electrophysiology Suites Using Master or Slave TTL Signals
- Tune Output Wavelength of Industry-Standard Tunable Ti:Sapphire Lasers

Data Analysis

- Assign a Color to Each Detection Channel
- Calculator Instantly Determines Image Dimensions and Resolution
- Generate 3D Z-Stack Reconstructions

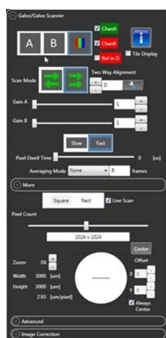
Capture Setup Tab

The ThorImageLS Capture Setup Tab offers a dedicated control panel for each module in your imaging rig. A selection of these panels is shown below.

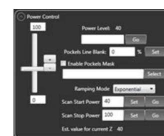


Click to Enlarge
Layout of Capture Setup Tab

Selected Capture Setup Tab Panels and Features



Click to Enlarge
Galvo-Galvo Scanner Control



Click to Enlarge
Laser Power Control

Galvo-Resonant or Galvo-Galvo Scanner Control
(Shown at Left)

- Choose Small Scan Areas for High Frame Rates or Large Scan Areas for High Resolution
- Line, Square, or Rectangular Scans
- Assign a Color to Each Detection Channel (Up to Four)
- Calculator Instantly Determines Pixel and Optical Resolution
- Change Pixel Dwell Time (of Galvo Axes) and Perform Frame Averaging

Laser Power Control
(Shown at Right)

- Exponential Power Ramping for Increasing Laser Power with Sample Depth
- Independently Control Power of All Input Lasers (Up to Four in Confocal Systems)
- Edge Blanking and Masking (Available with Pockels Cells)

Light Path Control
(Shown at Right)

- Insert and Remove Dichroic Mirrors for Different Scan Types
  - Epi-Fluorescence
  - Photoactivation/Uncaging
  - Widefield Illumination
  - Streaming Exposure
- Intuitive Layout Shows the Physical Arrangement of the Mirrors

Pinhole Control (Confocal Systems Only)
(Shown at Right)

- Select 1 of 16 Pinhole Diameters

- Align Pinhole to Boost Image's Signal-to-Noise

Click to Enlarge
Light Path Control

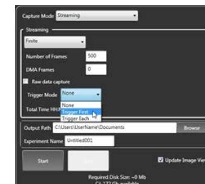
Click to Enlarge
Pinhole Control (Confocal Systems Only)

Capture Tab

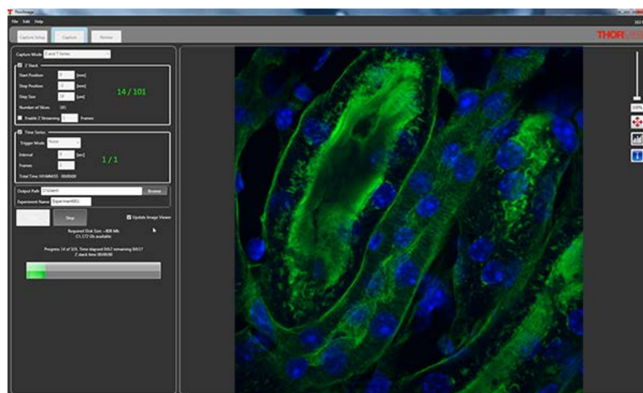
The ThorImageLS Capture Tab is a distraction-free area that keeps your focus on the collected data by only showing the parameters you need for the desired workflow. For example, in Streaming Mode (shown at right), it displays the option to acquire data immediately after clicking the Start button or to wait for an external stimulus. Contrast this to Bleaching Mode (also at right), which allows the user to set up different acquisition parameters for before and after the bleaching. In every workflow, the number of frames, scan duration, and required storage space are calculated and presented before each scan so that you know exactly what to expect.

All experimental data is saved in a lossless TIFF format for perfect fidelity. By choosing a standard image format, the images are viewable in ImageJ, Fiji, and many other image analysis programs, preventing lock-in to a specific program and preserving your data for the long term. ThorImageLS's Review Tab (see below) also provides quick and convenient analysis of finished acquisitions.

ThorImageLS directly supports dynamic scans, live streaming, image sequences triggered by a stimulus, and other modalities. Upon request, an SDK can be provided that permits custom acquisition sequences to be programmed by the user.


Click to Enlarge
Streaming Mode with Triggered Exposure

Click to Enlarge
Bleaching Mode

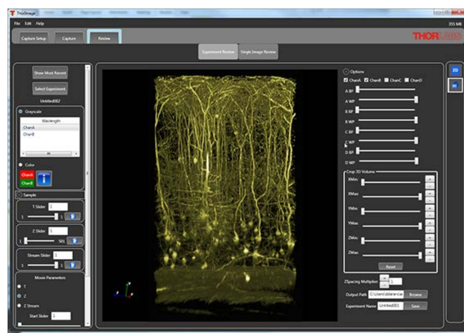

Click to Enlarge
Layout of Capture Tab

Review Tab

The ThorImageLS Review Tab lets you intuitively browse through previously acquired images, making it fast and easy to choose the exact image you want to analyze. Use sliders (shown below) to browse an acquisition sequence in time, visualize image planes along the Z-axis, or pick out one image from an entire stream.

Once you find what you're looking for, selectively enable and disable spectral channels to better visualize certain details of your specimen, or hover the mouse over the image to view the pixel's intensity (also shown below). The review tab also offers one-click 3D visualizations.

When you are ready to share your results, ThorImageLS's built-in movie maker will directly export the acquired Z series, time series, or image stream to AVI video.



Click to Enlarge
Layout of Review Tab
(Image Courtesy of Dr. Hajime Hirase and Katsuya Ozawa, RIKEN Brain Science Institute, Wako, Japan)

Click to Enlarge
Channel Color Selection and Image Sliders

Click to Enlarge
Intensity Readout

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Hide LSM Tutorial

LSM TUTORIAL

Laser Scanning Microscopy Tutorial

Laser scanning microscopy (LSM) is an indispensable imaging tool in the biological sciences. In this tutorial, we will be discussing confocal fluorescence imaging, multiphoton excitation fluorescence imaging, and second and third harmonic generation imaging techniques. We will limit our discussions to point scanning of biological samples with a focus on the technology behind the imaging tools offered by Thorlabs.

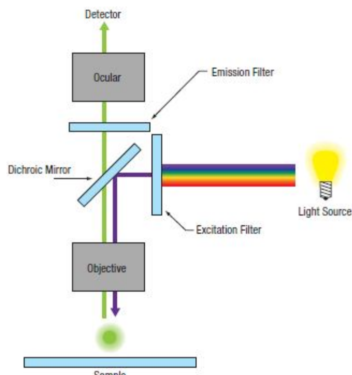
Introduction

The goal of any microscope is to generate high-contrast, high-resolution images. In much the same way that a telescope allows scientists to discern the finest details of the universe, a microscope allows us to observe biological functioning at the nanometer scale. Modern laser scanning microscopes are capable of generating multidimensional data (X, Y, Z, τ, λ), leading to a plethora of high-resolution imaging capabilities that further the understanding of underlying biological processes.

In conventional widefield microscopy (Figure 1, below left), high-quality images can only be obtained when using thin specimens (on the order of one to two cell layers thick). However, many applications require imaging of thick samples, where volume datasets or selection of data from within a specific focal plane is desired. Conventional widefield microscopes are unable to address these needs.

LSM, in particular confocal LSM and multiphoton LSM, allows for the visualization of thin planes from within a thick bulk sample, a technique known as optical sectioning. In confocal LSM, signals generated by the sample outside of the optical focus are physically blocked by an aperture, preventing their detection. Multiphoton LSM, as we will discuss later, does not generate any appreciable signal outside of the focal plane. By combining optical sectioning with incremented changes in focus (Figure 2, below right), laser scanning microscopy techniques can recreate 3D representations of thick specimen.

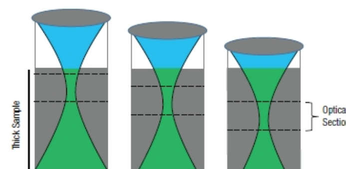
Figure 1 Widefield Epi-Fluorescence



Click to Enlarge

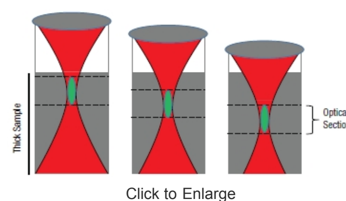
Figure 2 Optical Sections (Visualization of Thin Planes within a Bulk Sample)

Optical Sectioning in Confocal Microscopy



Click to Enlarge

Optical Sectioning in Multiphoton Microscopy



Click to Enlarge

Signal generated by the sample is shown in green. Optical sections are formed by discretely measuring the signal generated within a specific focal plane. In confocal LSM, out-of-focus light is rejected through the use of a pinhole aperture, thereby leading to higher resolution. In multiphoton LSM, signal is only generated in the focal volume. Signal collected at each optical section can be reconstructed to create a 3D image.

Contrast Mechanisms in LSM

Biological samples typically do not have very good contrast, which leads to difficulty in observing the boundaries between adjacent structures. A common method for improving contrast in laser scanning microscopes is through the use of fluorescence.

In fluorescence, a light-emitting molecule is used to distinguish the constituent of interest from the background or neighboring structure. This molecule can already exist within the specimen (endogenous or auto-fluorescence), be applied externally and attached to the constituent (chemically or through antibody binding), or transfected (fluorescent proteins) into the cell.

In order for the molecule to emit light (fluoresce) it must first absorb light (a photon) with the appropriate amount of energy to promote the molecule from the ground state to the excited state, as seen in Figure 3A below. Light is emitted when the molecule returns back down to the ground state. The amount of fluorescence is proportional to the intensity (I) of the incident laser, and so confocal LSM is often referred to as a linear imaging technique. Natural losses within this relaxation process require that the emitted photon have lower energy—that is, a longer wavelength—than the absorbed photon.

Multiphoton excitation (Figure 3B, below) of the molecule occurs when two (or more) photons, whose sum energy satisfies the transition energy, arrive simultaneously. Consequently, the two arriving photons will be of lower energy than the emitted fluorescence photon.

There are also multiphoton contrast mechanisms, such as harmonic generation and sum frequency generation, that use non-absorptive processes. Under conditions in which harmonic generation is allowed, the incident photons are simultaneously annihilated and a new photon of the summed energy is created, as illustrated in Figure 3C below.

Further constituent discrimination can be obtained by observing the physical order of the harmonic generation. In the case of second harmonic generation (SHG), signal is only generated in constituents that are highly ordered and lacking inversion symmetry. Third harmonic generation (THG) is observed at boundary interfaces where there is a refractive index change. Two-photon excitation and SHG are nonlinear processes and the signal generated is dependent on the square of the intensity (I^2).

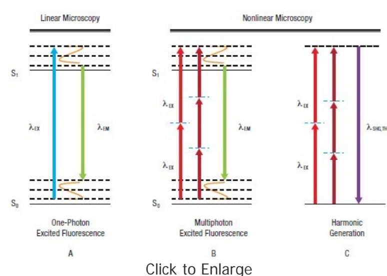
The nonlinear nature of signal generation in multiphoton microscopy means that high photon densities are required to observe SHG and THG. In order to accomplish this while maintaining relatively low average power on the sample, mode-locked femtosecond pulsed lasers, particularly Ti:Sapphire lasers, have become the standard.

Another consideration to be made in nonlinear microscopy is the excitation wavelength for a particular fluorophore. One might think that the ideal excitation wavelength is twice that of the one-photon absorption peak. However, for most fluorophores, the excited state selection rules are different for one- and two-photon absorption.

This leads to two-photon absorption spectra that are quite different from their one-photon counterparts. Two-photon absorption spectra are often significantly broader (can be >100 nm) and do not follow smooth semi-Gaussian curves. The broad two-photon absorption spectrum of many fluorophores facilitates excitation of several fluorescent molecules with a single laser, allowing the observation of several constituents of interest simultaneously.

All of the fluorophores being excited do not have to have the same excitation peak, but should overlap each other and have a common excitation range. Multiple fluorophore excitation is typically accomplished by choosing a compromising wavelength that excites all fluorophores with acceptable levels of efficiency.

Figure 3 Signal Generation in Laser Scanning Microscopy



Click to Enlarge

Absorptive Process (A, B):

The absorption of one or more excitation photons (λ_{EX}) promotes the molecule from the ground state (S_0) to the excited state (S_1). Fluorescence (λ_{EM}) is emitted when the molecule returns to the ground state.

Non-Absorptive Process (C):

The excitation photons (λ_{EX}) simultaneously convert into a single photon ($\lambda_{SHG,THG}$) of the sum energy and half (for SHG) or one-third (for THG) the wavelength.

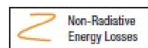


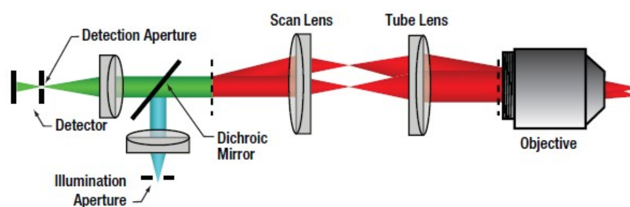
Image Formation

In a point-scanning LSM, the single-plane image is created by a point illumination source imaged to a diffraction-limited spot at the sample, which is then imaged to a point detector. Two-dimensional *en face* images are created by scanning the diffraction-limited spot across the specimen, point by point, to form a line, then line by line in a raster fashion.

The illuminated volume emits a signal which is imaged to a single-element detector. The most common single-element detector used is a photomultiplier tube (PMT), although in certain cases, avalanche photodiodes (APDs) can be used. CCD cameras are not typically used in point-scanning microscopes, though are the detector of choice in multifocal (i.e. spinning disk confocal) applications.

The signal from the detector is then passed to a computer which constructs a two-dimensional image as an array of intensities for each spot scanned across the sample. Because no true image is formed, LSM is referred to as a digital imaging technique. A clear advantage of single-point scanning and single-point detection is that the displayed image resolution, optical resolution, and scan field can be set to match a particular experimental requirement and are not predefined by the imaging optics of the system.

Figure 4 Confocal Optical Path



Confocal LSM

In confocal LSM, point illumination, typically from a single mode, optical-fiber-coupled CW laser, is the critical feature that allows optical sectioning. The light emitted from the core of the single mode optical fiber is collimated and used as the illumination beam for scanning. The scan system is then imaged to the back aperture of the objective lens which focuses the scanned beam to a diffraction-limited spot on the sample. The signal generated by the focused illumination beam is collected back through the objective and passed through the scan system.

After the scan system, the signal is separated from the illumination beam by a dichroic mirror and brought to a focus. The confocal pinhole is located at this focus. In this configuration, signals that are generated above or below the focal plane are blocked from passing through the pinhole, creating the optically sectioned image (Figure 2, above). The detector is placed after the confocal pinhole, as illustrated in Figure 4 to the right. It can be inferred that the size of the pinhole has direct consequences on the imaging capabilities (particularly, contrast, resolution and optical section thickness) of the confocal microscope.

The lateral resolution of a confocal microscope is determined by the ability of the system to create a diffraction-limited spot at the sample. Forming a diffraction-limited spot depends on the quality of the laser beam as well as that of the scan optics and objective lens.

The beam quality is typically ensured by using a single mode optical fiber to deliver the excitation laser light as a Gaussian point source, which is then collimated and focused into a diffraction-limited beam. In an aberration-free imaging system, obtained by using the highest quality optical elements, the size of this focus spot, assuming uniform illumination, is a function of excitation wavelength (λ_{EX}) and numerical aperture (NA) of the objective lens, as seen in Equation 1.

$$\text{Spot Size} = \frac{1.22\lambda_{EX}}{\text{NA}}$$

Equation 1 Spot Size

In actuality, the beam isn't focused to a true point, but rather to a bullseye-like shape. The spot size is the distance between the zeros of the Airy disk (diameter across the middle of the first ring around the center of the bullseye) and is termed one Airy Unit (AU). This will become important again later when we discuss pinhole sizes.

The lateral resolution of the imaging system is defined as the minimum distance between two points for them to be observed as two distinct entities. In confocal (and multiphoton) LSM, it is common and experimentally convenient to define the lateral resolution according to the full width at half maximum (FWHM) of the individual points that are observed.

Using the FWHM definition, in confocal LSM, the lateral resolution ($R_{\text{lateral,confocal}}$) is:

$$R_{\text{lateral,confocal}} = \frac{0.51\lambda_{EX}}{\text{NA}}$$

Equation 2 Lateral Resolution, Confocal LSM

and the axial resolution ($R_{\text{axial,confocal}}$) is:

$$R_{\text{axial,confocal}} = \frac{0.88\lambda_{EX}}{n - \sqrt{n^2 - (\text{NA})^2}}$$

Equation 3 Axial Resolution, Confocal LSM

where *n* is the refractive index of the immersion medium.

It is interesting to note that in a confocal microscope, the lateral resolution is solely determined by the excitation wavelength. This is in contrast to widefield microscopy, where lateral resolution is determined only by emission wavelength.

To determine the appropriate size of the confocal pinhole, we multiply the excitation spot size by the total magnification of the microscope:

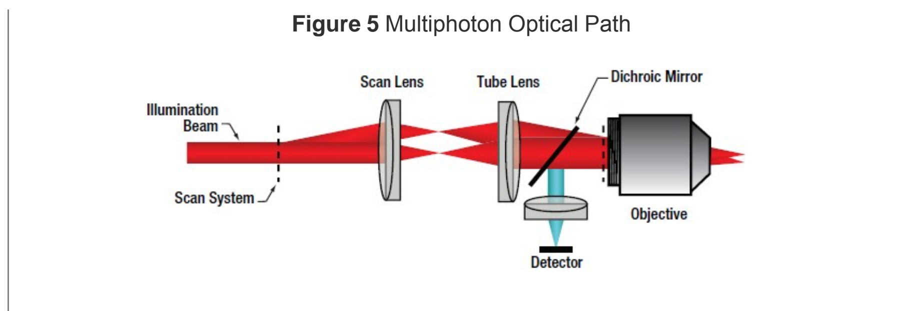
$$D_{\text{pinhole}} = M_{\text{objective}} \times M_{\text{scan head}} \times \text{Spot Size}$$

Equation 4 Pinhole Diameter

As an example, the appropriate size pinhole for a 60X objective with NA = 1.0 for λ_{EX} = 488 nm ($M_{\text{scan head}}$ = 1.07 for the Thorlabs Confocal Scan Head) would be 38.2 µm and is termed a pinhole of 1 AU diameter. If we used the same objective parameters but changed the magnification to 40X, the appropriate pinhole size would be 25.5 µm and would also be termed a pinhole of 1 AU diameter. Therefore, defining a pinhole diameter in terms of AU is a means of normalizing pinhole diameter, even though one would have to change the pinhole selection for the two different objectives.

Theoretically, the total resolution of a confocal microscope is a function of the excitation illumination spot size and the detection pinhole size. This means that the resolution of the optical system can be improved by reducing the size of the pinhole. Practically speaking, as we restrict the pinhole diameter, we improve resolution and confocality, but we also reduce the amount of signal reaching the detector. A pinhole of 1 AU is a good balance between signal strength, resolution, and confocality.

Figure 5 Multiphoton Optical Path



Multiphoton LSM

In multiphoton LSM, a short pulsed free-space laser supplies the collimated illumination beam that passes through the scanning system and is focused by the objective. The very low probability of a multiphoton absorption event occurring, due to the I^2 dependence of the signal on incident power, ensures signal is confined to the focal plane of the objective lens. Therefore, very little signal is generated from the regions above and below the focal plane. This effective elimination of out-of-focus signal provides inherent optical sectioning capabilities (Figure 2, above) without the need for a confocal pinhole. As a result of this configuration, the collected signal does not have to go back through the scanning system, allowing the detector to be placed as close to the objective as possible to maximize collection efficiency, as illustrated in Figure 5 to the right. A detector that collects signal before it travels back through the scan system is referred to as a non-descanned detector.

Again using the FWHM defintion, in multiphoton LSM, the lateral resolution ($R_{\text{lateral,multiphoton}}$) is:

$$R_{\text{lateral,multiphoton,NA}>0.7} = \frac{0.383\lambda_{EX}}{(\text{NA})^{0.91}}$$

Equation 5 Lateral Resolution, Multiphoton LSM

and the axial resolution ($R_{\text{axial,multiphoton}}$) is:

$$R_{\text{axial,multiphoton,NA}>0.7} = \frac{0.626\lambda_{EX}}{n - \sqrt{n^2 - (\text{NA})^2}}$$

Equation 6 Axial Resolution, Multiphoton LSM

These equations assume an objective NA > 0.7, which is true of virtually all multiphoton objectives.

The longer wavelength used for multiphoton excitation would lead one to believe (from Equation 5) that the resolution in multiphoton LSM, compared to confocal LSM, would be reduced roughly by a factor of two. For an ideal point object (i.e. a sub-resolution size fluorescent bead) the I^2 signal dependence reduces the effective focal volume, more than offsetting the 2X increase in the focused illumination spot size.

We should note that the lateral and axial resolutions display a dependence on intensity. As laser power is increased, there is a corresponding increase in the probability of signal being generated within the diffraction-limited focal volume. In practice, the lateral resolution in a multiphoton microscope is limited by how tightly the illumination beam can be focused and is well approximated by Equation 5 at moderate intensities. Axial resolution will continue to degrade as excitation power is increased.

Image Display

Although we are not directly rendering an image, it is still important to consider the size of the image field, the number of pixels in which we are displaying our image (capture resolution) on the screen, and the lateral resolution of the imaging system. We use the lateral resolution because we are rendering an *en face* image. In order to faithfully display the finest features the optical system is capable of resolving, we must appropriately match resolution (capture and lateral) with the scan field. Our capture resolution must, therefore, appropriately sample the optical resolution.

In LSM, we typically rely on Nyquist sampling rules, which state that the pixel size should be the lateral resolution divided by 2.3. This means that if we take our 60X objective from earlier, the lateral resolution is 249 nm (Equation 2) and the pixel size in the displayed image should be 108 nm. Therefore, for a 1024 x 1024 pixel capture resolution, the scan field on the specimen would be ~111 µm x 111 µm. It should be noted that the 40X objective from our previous example would yield the exact same scan field (both objectives have the same NA) in the sample. The only difference between the two images is the angle at which we tilt our scanners to acquire the image.

It may not always be necessary to render images with such high resolution. We can always make the trade-off of image resolution, scan field, and capture resolution to create a balance of signal, sample longevity, and resolution in our images.

Considerations in Live Cell Imaging

One of LSM's greatest attributes is its ability to image living cells and tissues. Unfortunately, some of the by-products of fluorescence can be cytotoxic. As such, there is a delicate balancing act between generating high-quality images and keeping cells alive.

One important consideration is fluorophore saturation. Saturation occurs when increasing the laser power does not provide the expected

concurrent increase in the fluorescence signal. This can occur when as few as 10% of the fluorophores are in the excited state.

The reason behind saturation is the amount of time a fluorophore requires to relax back down to the ground state once excited. While the fluorescence pathways are relatively fast (hundreds of ps to a few ns), this represents only one relaxation mechanism. Triplet state conversion and nonradiative decay require significantly longer relaxation times. Furthermore, re-exciting a fluorophore before it has relaxed back down to the ground state can lead to irreversible bleaching of the fluorophore. Cells have their own intrinsic mechanisms for dealing with the cytotoxicity associated with fluorescence, so long as excitation occurs slowly.

One method to reduce photobleaching and the associated cytotoxicity is through fast scanning. While reducing the amount of time the laser spends on a single point in the image will proportionally decrease the amount of detected signal, it also reduces some of the bleaching mechanisms by allowing the fluorophore to completely relax back to the ground state before the laser is scanned back to that point. If the utmost in speed is not a critical issue, one can average several lines or complete frames and build up the signal lost from the shorter integration time.

The longer excitation wavelength and non-descanned detection ability of multiphoton LSM give the ability to image deeper within biological tissues. Longer wavelengths are less susceptible to scattering by the sample because of the inverse fourth power dependence (l^{-4}) of scattering on wavelength. Typical penetration depths for multiphoton LSM are 250 - 500 µm, although imaging as deep as 1 mm has been reported in the literature, compared to ~100 µm for confocal LSM.

Thorlabs recognizes that each imaging application has unique requirements.
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Hide Part Numbers

Part Number	Description	Price	Availability
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MPM-SCAN4	NIR Galvo-Resonant Scan Head with 4-Channel Acquisition	$50,000.00	Lead Time