

## CLS-2HS - June 15, 2015

Item # CLS-2HS was discontinued on June 15, 2015. For informational purposes, this is a copy of the website content at that time and is valid only for the stated product.

### CONFOCAL MICROSCOPE



[Hide Overview](#)

#### OVERVIEW

##### Thorlabs' Confocal Microscope

- Designed to Convert Research-Grade Microscopes to Confocal Imaging Systems
- Compatible with Upright or Inverted Microscopes
- Compact, Modular Design
- Video-Rate Image Acquisition:  
512 x 512 Pixels at 30 fps
- Capture Resolution
  - 2048 x 2048 Pixels (Bi-Directional)
  - 4096 x 4096 Pixels (Uni-Directional)



Thorlabs' Confocal Laser Scanning (CLS) Microscopy Systems are compact imaging modules designed to bring powerful confocal imaging tools within the reach of any research lab. By eliminating signals that originate from outside the focal plane, confocal microscopy provides the ability to acquire high resolution, optically sectioned images from within a thick sample or to reduce background fluorescence from thin cultures. The CLS systems offer turnkey integration with virtually any upright or inverted microscope (not included) with access to the intermediate image plane (e.g., camera port) via a C-Mount threading. The included ThorImageLS™ software drives the CLS hardware via an intuitive graphical user interface, providing quick data recording and review.

Our basic Confocal Laser Scanning System includes an electronics control unit, an expandable dual PMT module, a 16-position pinhole wheel, a two-channel (488 nm & 642 nm) laser source, and the ThorImageLS™ acquisition software and computer workstation. For more sophisticated imaging requirements, systems are available with up to four laser lines and four high-sensitivity GaAsP PMTs.

##### Confocal System Components

- Galvo-Resonant Scan Head
  - Provides Video Rate Image Acquisition:  
512 x 512 Pixels at 30 fps
  - 400 fps at 512 x 32 Pixels
  - Maximum Scan Resolution of 4096 x 4096 Pixels
- Standard Multi-alkali or High-Sensitivity GaAsP PMTs
- Fiber-Coupled Laser Source with Up to Four Excitation Wavelengths (See the *Specs* Tab for Wavelength Options)
- 16-Position Motorized Pinhole Wheel
- ThorImageLS™ Software Suite for Automated Data Collection
- Confocal Z-Stepper Motor (Available Separately)

A 4-channel confocal microscopy system is shown mounted on a Nikon FN1 in the video to the right. This system features a 4-wavelength integrated laser source, a 4-channel detection module with standard sensitivity PMTs. The galvo-resonant scan head has two fiber inputs and has the 16 position motorized pinhole wheel mounted to the side. The full range of system configurations, including laser source and emission

filter options, are outlined on the *Confocal System* tab. For system specifications, please see the *Specs* 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](mailto:ImagingSales@thorlabs.com) or call (703) 651-1700.

[Hide Confocal System](#)

## CONFOCAL SYSTEM

### Thorlabs' Confocal Microscopy System

Thorlabs' Confocal Laser Scanning (CLS) Microscopy Systems consist of compact imaging modules specifically designed for infinity-corrected compound microscopes. They provide the ability to acquire high-resolution optical sections from within a thick sample or to reduce background fluorescence from a thin culture. The CLS systems offer turnkey integration to almost any upright or inverted microscope with access to an intermediate image plane (e.g., a camera port) via a C-Mount threading. The included software has an intuitive graphical interface that allows data to be quickly recorded and reviewed while providing sophisticated peripheral controls for image acquisition. CLS systems are user-installable, although on-site installation is also available.



Click to Enlarge  
Thorlabs' Confocal Microscopy System mounted on an inverted microscope.

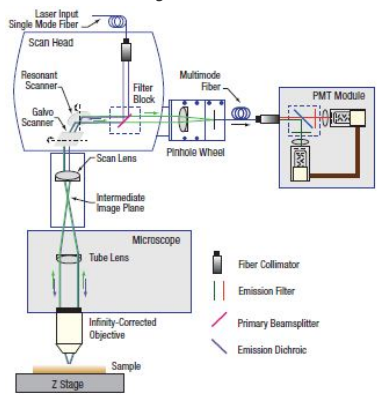
All hardware components are directly controlled by the ThorImageLS™ software, including automated Z-step control for optical sectioning (via a piezo or stepper motor) and automatic calculation of Airy disk units based on the combination of the objective magnification and pinhole size. Our intuitive interface allows novice and experienced users alike to obtain high-resolution microscope images quickly and easily.

All complete CLS systems include a multi-channel fiber-coupled laser source, control electronics, scan head, pinhole wheel, detectors, and all fibers and cables needed to interconnect the system.

Additionally, each system includes a Windows® computer with a 24" monitor, data acquisition hardware, and control boards as well as the ThorImageLS software.

Thorlabs' applications engineers install each confocal system and are available to address technical problems that may occur. We also include a comprehensive installation and operation manual with basic preventative maintenance instructions to ensure that your system performs optimally for years to come. Also available are complete systems that combine the Thorlabs Confocal package with third party upright and inverted microscopes. For further details on this convenient option, please contact us at [ImagingSales@thorlabs.com](mailto:ImagingSales@thorlabs.com).

### Confocal System Schematic



Click to Enlarge



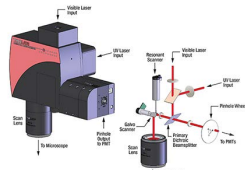
Click to Enlarge  
The scan head of Thorlabs' Confocal System.

### Scanner

At the heart of our systems is an efficiently designed Scan Head that incorporates an 8 kHz resonant scanner and a galvanometer for fast image acquisition. This allows for high imaging speeds of up to 400 frames per second (at 512 x 32 pixel resolution) or images with high spatial resolution of up to 4096 x 4096 pixel resolution (at 2 fps). At either extreme, or anywhere in-between, the control and acquisition system creates high-quality, jitter-free images.

The confocal scan head module consists of three principal components: the high-speed resonant scanner, a wide field of view scan lens, and a pinhole wheel output. The scanner can be attached to virtually any upright or inverted microscope with access to the

intermediate image plane via a C-mount-threaded port (e.g., a camera port) as long as 100% of the output can be directed to this port. As shown in the drawing to the right, light enters the scan head through one of the laser inputs, is reflected onto the galvo-resonant scanner mirrors by the primary filter block, and exits the assembly through the wide field of view scan lens. Light then enters the microscope and excites fluorophores in the sample. The resulting fluorescence travels back through the scan lens and is reflected by the galvo-resonant scanner to the pinhole wheel. The pinhole wheel output is connected to the PMT detection module via an SMA-terminated fiber.



Click to Enlarge  
A diagram showing the light path through the confocal scan head.

Our complete systems come standard with a primary dichroic that reflects four laser lines (405, 488, 532, and 642 nm). Other primary dichroics for use with other wavelengths can be provided upon request.

### Wide Field to View Scan Lens

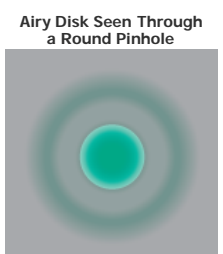
To complement the large angular range over which the resonant scanner is used, Thorlabs' engineers developed a scan lens optimized for large fields of view. The lens features excellent chromatic aberration correction from 405 to 1100 nm (antireflective coating effective from 405 to 750 nm), superb field flatness, and very low distortion the field of view. This broad wavelength range adds to the functionality of the system by enabling the use of laser sources down to 400 nm while color correcting fluorescence emissions from even the deepest of red-emitting fluorophores. Coupled with ultra-sensitive, low-noise detectors and control electronics, we are able to provide systems which redefine the boundaries of contrast, resolution, and imaging speed at an affordable cost.

### Motorized Pinhole Wheel

The motorized pinhole wheel (also available separately as the MPH16) allows the pinhole size to be adjusted for a variety of

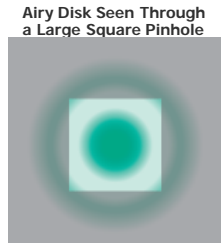


Airy Disk



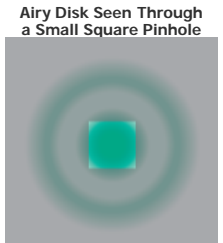
Airy Disk Seen Through a Round Pinhole

A round pinhole cleanly isolates the in-focus light and blocks out-of-focus light.



Airy Disk Seen Through a Large Square Pinhole

A large square pinhole superscribed on the Airy Disk allows all of the in-focus light to pass, but some of the out-of-focus light will leak through as well.



Airy Disk Seen Through a Small Square Pinhole

A small square pinhole inscribed on the peak of the Airy Disk blocks out-of-focus light, but also eliminates some of the usable signal.

imaging configurations and objective numerical apertures in order to simultaneously maximize the in-focus light that reaches the PMT detectors and minimize the transmission of signal from outside the focal plane. A rotating glass plate has 16 sizes of lithographic round pinholes deposited to exceedingly tight tolerances, ensuring that optimal alignment is maintained as each pinhole is rotated into the light path.

For thicker samples, the size of the pinhole should be optimized relative to the NA of the objective in order to maximize signal to noise. With this in mind, our engineers selected each pinhole size to complement a common objective NA. Conversely, for thinner samples that produce less light outside of the focal plane, a larger pinhole size can help improve throughput. Pinhole diameters up to Ø2 mm provide flexibility so that the system can be easily adapted the system to different experiments.



Click to Enlarge  
The motorized filter wheel shown disconnected from the confocal scan head with the SMA fiber connector removed for free-space output.

The diagram to the right illustrates the effects of several common pinhole shapes on the signal that reaches your detector. A round pinhole is the ideal shape for maximizing the transmission of light generated in the focal plane of your sample while also optimizing the rejection of signal generated above and below the layer that is being scanned.

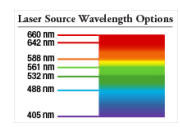
The pinhole is conveniently powered and controlled over USB. Additionally, the motorized, encoded control of the pinhole ensures perfect alignment and vibration-free movement. The emitted light from the specimen is focused on the pinhole and then collected by a large-core multimode fiber for transmission to the PMT detector system. More details on our motorized pinhole are available here.



Click to Enlarge  
Four-Channel Laser Source

**Excitation**

The solid state multilaser source minimizes maintenance with an all-fiber design. Each laser line is individually fiber-coupled using a permanent rigid system. The individual fiber-coupled lasers are then combined in an all-fiber coupler. This design ensures the lasers never go out of alignment by keeping the full power of the lasers coupled to the scan head at all times. For added flexibility, a second fiber output is provided that is dedicated to an optional 405 nm laser diode.



The combined visible output is contained in a single mode fiber with an FC/PC connector. The optional 405 nm laser output is delivered on its own single mode fiber and is combined after the beam expander in the Scan Head module. By combining the 405 nm light after the beam expander, we are able to couple the UV laser into the lightpath with a 4 mm beam diameter, which increases stability and maintains the color correction of the system.

Each confocal system also includes a filter set, chosen to complement the excitation wavelengths. Available pre-configured laser source wavelength combinations and the included filter sets are outlined in the table below.

The entire laser source is controlled by a single USB connection, which allows the user to turn each laser on and off as well as control its intensity.

Laser Source Options						
Laser Source # <sup>a</sup>	Excitation Wavelengths				Included Emission Filters	
	UV	Blue	Green/Orange	Red	Emission Filters (Center Wavelength/Bandwidth)	Longpass Dichroic Cutoff Wavelength(s)
CMLS-A	-	488 nm	-	642 nm	525 nm/45 nm and 635 nm/Longpass	562 nm
CMLS-B <sup>b</sup>	405 nm	488 nm	-	642 nm	447 nm/60 nm, 512/25 nm, and 635 nm/Longpass	495 nm and 538 nm
CMLS-C	-	488 nm	532 nm	642 nm	513 nm/17 nm, 582 nm/75 nm, and 635 nm Longpass	538 nm and 649 nm
CMLS-D	-	488 nm	561 nm	642 nm	525 nm/45 nm, 607 nm/36 nm, and 535 nm/Longpass	562 nm and 649 nm
CMLS-E <sup>b</sup>	405 nm	488 nm	532 nm	642 nm	447 nm/60 nm, 513 nm/17 nm, 582 nm/75 nm, and 635 nm/Longpass	495 nm, 538 nm, and 649 nm
CMLS-F <sup>b</sup>	405 nm	488 nm	561 nm	642 nm	447 nm/60 nm, 525 nm/45 nm, 607 nm/36 nm, and 635 nm/Longpass	495 nm, 562 nm, and 469 nm
CMLS-G <sup>b</sup>	405 nm	488 nm	588 nm	642 nm	447 nm/60 nm, 525 nm/45 nm, 615 nm/24 nm, and 635 nm/Longpass	495 nm, 605 nm, and 649 nm
CMLS-H	-	488 nm	-	660 nm	525 nm/39 nm and 697 nm/58 nm	562 nm
CMLS-I	-	488 nm	-	-	525 nm/45 nm	N/A
CMLS-J	-	488 nm	532 nm	-	512 nm/25 nm and 582 nm/75 nm	538 nm

a. For sources with less than four lasers, slots will be filled from left to right  
b. These sources are only compatible with a scan head that has a second fiber input port for the UV.



Click to Enlarge  
Expandable PMT modules are designed for multi-channel laser scanning microscopy applications. The photo shows the 4-channel module with standard sensitivity PMTs.



Click to Enlarge  
The filter sets are mounted in DFM filter cubes for easy exchange. Here, an emission filter is being removed from a 2-channel PMT module with high-sensitivity PMTs.

## Detection

**Detector:** Future-proof your experiments with our remotely positioned detector module that can be readily expanded from two to four photomultiplier tubes (PMTs). Sitting in front of each PMT is a quickly exchangeable dichroic mirror and emission filter holder (for included filter sets, see the table above).



Click to Enlarge  
Each Confocal System comes with either a Dell (32 bit or 64 bit) or Superlogics (64 bit) computer tower with ThorImageLS installed and a 24" monitor for data acquisition and review.

The detection module can be configured with our standard sensitivity multi-alkali PMTs or high sensitivity, ultra-low-noise GaAsP PMTs. The standard sensitivity multi-alkali PMTs provide a low-noise image with high dynamic range that is appropriate for most life-science and industrial applications. For weakly fluorescent or highly photosensitive samples, we also offer the option of high-sensitivity, TEC-cooled GaAsP PMTs. With either choice, the gain of the detector as well as the dynamic range of the digitizer is controlled within the ThorImageLS software.

**Filters:** Each confocal system includes an appropriate set of emission filters that block laser light from entering the PMTs and provide pass bands at the fluorescence wavelengths of popular fluorophores. The exact configuration is determined by the laser wavelengths in your confocal system. Several common configurations and compatible fluorophores are outlined below. If you have questions concerning the filter set included with a specific laser configuration, please contact [ImagingSales@thorlabs.com](mailto:ImagingSales@thorlabs.com) for more information.

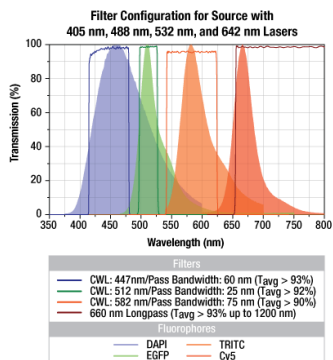
**Computer:** Each confocal system includes a computer with a 24" monitor. Depending on your confocal system configuration, the system can be controlled using a 32-bit or 64-bit Dell computer. For systems requiring higher computational power, we also offer a Superlogics tower. Each computer comes with the ThorImageLS software package installed, providing an all-in-one solution for microscope control, automated data collection, and image review.

## Sample Filter Configurations

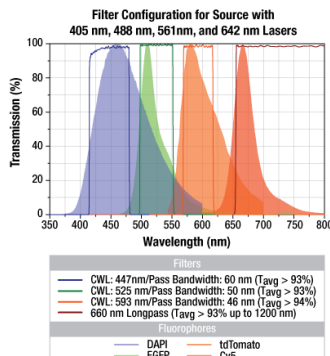
In addition to the filter set list above, the two sample plots of emission filter pass bands are provided below as an additional example of how Thorlabs' Confocal System can work with popular fluorophores.

The primary dichroic and emission filter sets in the confocal system are typically optimized for one of two excitation wavelength configurations. The most popular configuration is compatible with 405 nm, 488 nm, 561 nm, and 642 nm excitation lasers. The graph to the lower left provides an example of this common emission filter configuration, with the emission spectra of four compatible fluorophores superimposed on the filters' transmission profiles.

The second most common 4-laser configuration exchanges the 561 nm laser for a 562 nm laser, useful for samples that are marked with TRITC. To accommodate this wavelength, the two bandpass filters centered at 525 nm and 593 nm would be replaced with a narrower bandpass filter (25 nm pass bandwidth) centered at 512 nm and a wider bandpass filter (75 nm pass bandwidth) centered at 582 nm.



Click to Enlarge  
The solid lines in the plot show the pass bands of the filters, and the shaded areas show the normalized emission spectrum for the fluorophores.



Click to Enlarge  
The solid lines in the plot show the pass bands of the filters, and the shaded areas show the normalized emission spectrum for the fluorophores.

## Thorlabs' Confocal Microscopy System



The photo above shows a confocal system coupled to an upright microscope on one of our Nexus optical tables (not included).

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

## S P E C S

The Confocal system Item #'s in this table represent popular confocal configurations. Additional laser sources are available and are listed with the included emission filter sets in the second table below. A description of the modules that make up each confocal system is provided on the *Confocal System* tab. For more information, contact our sales team and applications engineers at [ImagingSales@thorlabs.com](mailto:ImagingSales@thorlabs.com) or (701) 651-1700.

		CLS-2SS	CLS-2HS	CLS-4SS	CLS-4HS	CLS-UV
<b>Excitation</b>						
Laser Wavelengths for Pre-Configured Systems (Additional Combinations Available Upon Request)	405 nm			x	x	x
	488 nm	x	x	x	x	x
	532 nm			x	x	
	642 nm	x	x	x	x	x
Primary Dichroic Mirror		Quad Band Dichroic Beamsplitter				
<b>Scanning</b>						
Scan Head		Galvo-Resonant Scan Head with 8 kHz Resonant Scanner (X) and Galvo Scan Mirror (Y)				
Galvo-Resonant Scanning Speed		30 Frames per Second at 512 x 512 Pixels 400 Frames per Second at 512 x 32 Pixels 2 Frames per Second at 4096 x 4096 Pixels				
Scan Zoom		Up to 2048 x 2048 Bi-Directional Acquisition; Up to 4096 x 4096 Uni-Directional Acquisition 1X - 16X (Continuous)				
Capture Resolution		Up to 2048 x 2048 Bi-Directional Acquisition; Up to 4096 x 4096 Uni-Directional Acquisition				
Diffraction-Limited Field of View (FOV)		FN25 = 442 µm x 442 µm FOV @ 40X; FN23 = 407 µm x 407 µm FOV @ 40X				
<b>Emission</b>						
Number of PMTs Included	Standard Sensitivity	2		4		2
	High Sensitivity		2		4	
Secondary Dichroic (CWL)	495 nm			x	x	x
	562 nm	x	x	x	x	x
	605 nm <sup>a</sup> or 649 nm <sup>b</sup>			x	x	

a. Filters when using the 532 nm laser.

b. Filters when using the 561 nm laser.

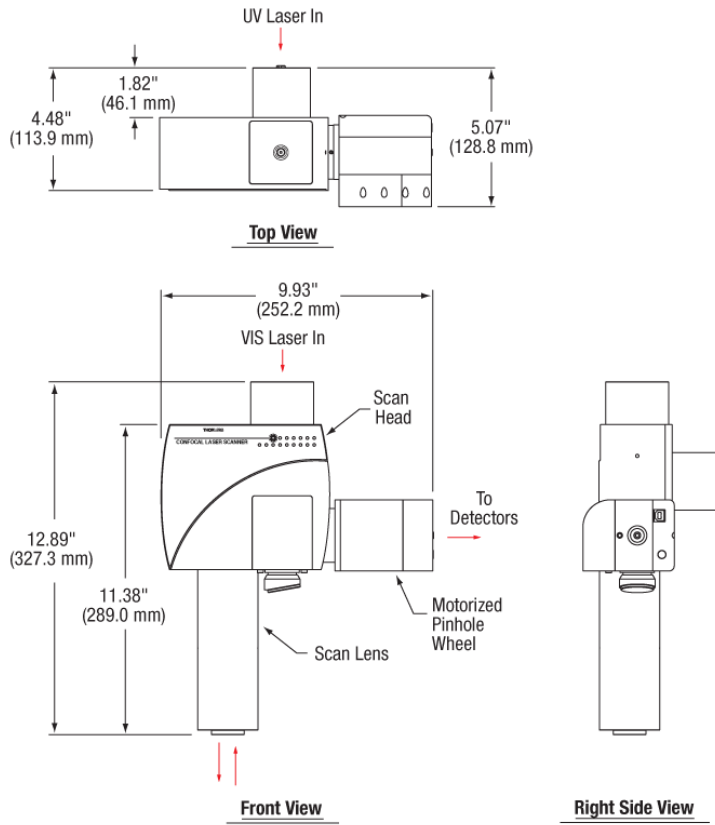
The laser wavelengths offered as part of the confocal system are available in in one of 10 pre-configured integrated laser sources. Each source is paired with a set of filters optimized for popular fluorophores that can be mounted in the PMT detection module. If you need help selecting a laser source for you system, you can contact our applications engineers by e-mail at [ImagingSales@thorlabs.com](mailto:ImagingSales@thorlabs.com) or by calling (701) 651-1700.

Laser Source Options						
Laser Source # <sup>a</sup>	Excitation Wavelengths				Included Emission Filters	
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CMLS-D	-	488 nm	561 nm	642 nm	525 nm/45 nm, 607 nm/36 nm, and 535 nm/Longpass	562 nm and 649 nm
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CMLS-I	-	488 nm	-	-	525 nm/45 nm	N/A
CMLS-J	-	488 nm	532 nm	-	512 nm/25 nm and 582 nm/75 nm	538 nm

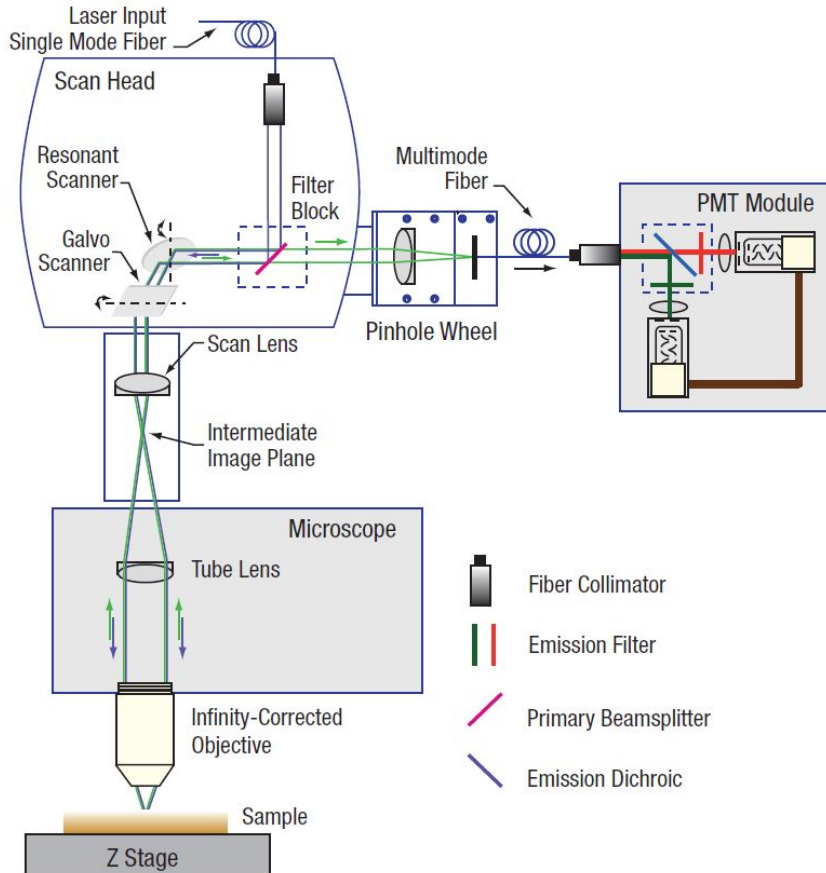
a. The laser source is not offered separately from the Confocal System, but we have provided a laser source # hear for ease of identification when discussing a system configuration with one of our representatives. For sources with less than four lasers, slots will be filled from left to right.

b. These sources are only compatible with a scan head that has a second fiber input port for the UV.

### Scan Head Dimensions



### System Schematic



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## SOFTWARE

**ThorImageLS: Intuitive Workflow-Oriented Software Suite**

ThorImageLS™ was developed side-by-side with our multiphoton and confocal 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.



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 even the wavelength of a tunable Coherent Chameleon™ Ti:Sapphire laser. This high degree of automation minimizes distractions, allowing you to keep your focus on your research.

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

**Experimental Techniques**

- Ramp Power with Sample Depth to Minimize Damage While Maximizing Deep Signal-to-Noise
- Customize Acquisition Parameters for High-Speed Z-Stacks or Image Streams
- Select Region of Interest to Photoactivate/Bleach with an Easy-to-Use Interface

**Equipment Control**

- Control Power of Independent Excitation Lasers
- Insert/Remove Dichroic Mirrors for Different Scan Types
- Integrate with Electrophysiology Suites Using Master or Slave TTL Signals
- Tune Output Wavelength of Coherent Chameleon™ Ti:Sapphire Lasers

**Data Analysis**

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

**Download**

Please contact [ImagingTechSupport@thorlabs.com](mailto:ImagingTechSupport@thorlabs.com) to obtain the latest ThorImageLS version compatible with your microscope. Because ThorImageLS 2.x adds significant new features over 1.x versions, it is not compatible with older microscopes. We are continuing to support version 1.5 for customers with older hardware.

[Contact Us](#)

**Version 2.2** - February 26, 2015*New Features*

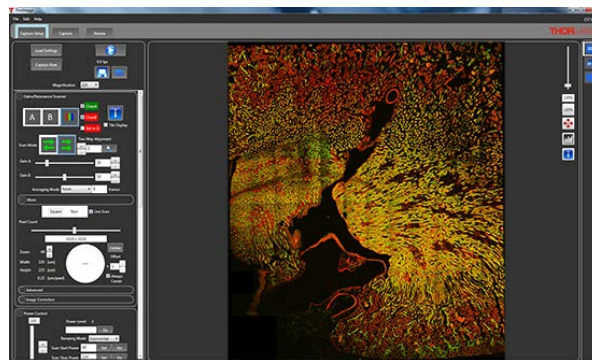
- Added capability for adding multiple ROIs with statistics
- Added capability for multi-point bleaching (requires galvo-galvo scanner)
- Added real-time ROI statistics calculations during capture
- Added ROI statistics table and chart plotter to Review tab
- Added option for turning on resonant scanner at software startup
- Added feature to set X and Y stage positions to "0", similar to Z function
- Added feature to select which Z position image is displayed during fast Z imaging
- Added capability to increase frame rate capture to 8 FPS

*Fixed Bugs*

- Negative value for galvo-galvo two-way offset shifts image incorrectly
- Y offset for galvo-resonant line mode is always 0 V
- ThorImageLS crash if Windows' "User Folder" path points to network location
- ThorImageLS crash if entering a value greater than 255 for field size
- Galvo-resonant line scan not working if Pockels is configured
- Tooltips for galvo-galvo slow and fast dwell time buttons are incorrect
- Pockels not working correctly for galvo-galvo if dwell time  $\leq 1 \mu\text{s}$
- Galvo-galvo trigger first feature not functional for time series
- Can't enter a negative value in Pockels calibration minimum value field
- Galvo-galvo detector polarity setting not reading from correct settings file

**Capture Setup Tab**

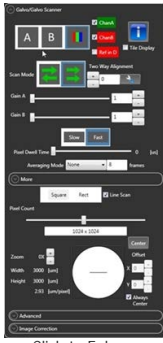
The ThorImageLS Capture Setup Tab offers a dedicated control panel for each module in your imaging rig. A selection of these panels is shown below.



Click to Enlarge  
Layout of Capture Setup Tab



## Selected Capture Setup Tab Panels and Features



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Galvo-Galvo Scanner 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)

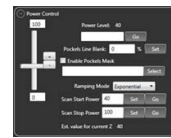
- Exponential Power Ramping for Increasing Laser Power with Sample Depth
- Independently Control Power of All Input Lasers (Up to Four in Confocal Systems)
- Edge Blanking and Masking (Available with Pockels Cells)

### 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  
Laser Power Control



Click to Enlarge  
Light Path Control



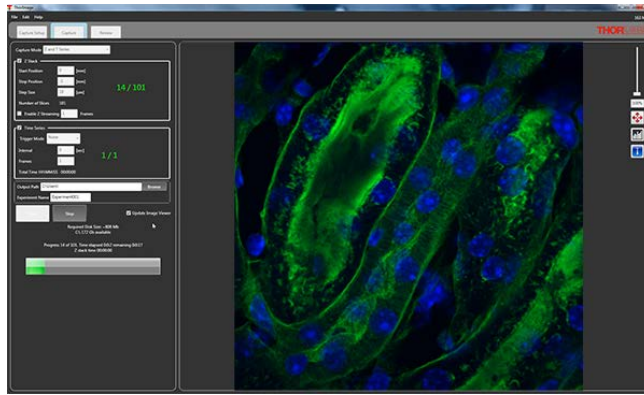
Click to Enlarge  
Pinhole Control (Confocal  
Systems Only)

## 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.



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Layout of Capture Tab



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Streaming Mode with Triggered Exposure



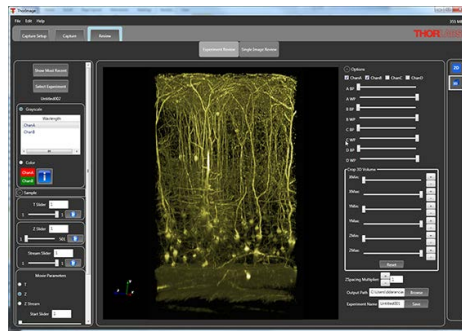
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Bleaching Mode

## 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.



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



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## 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.

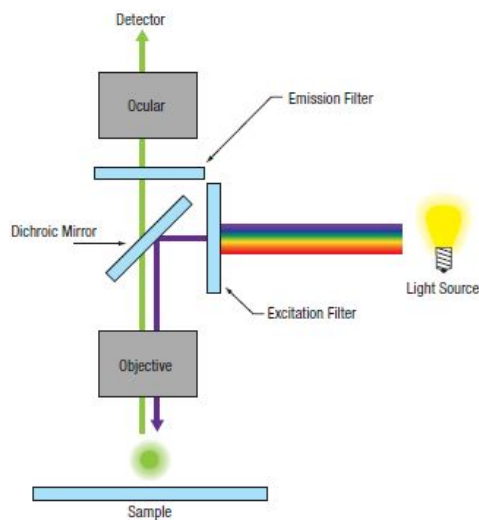
### Introduction

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,  $\tau$ ,  $\lambda$ ), 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.

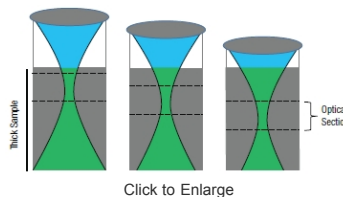
LSM, in particular confocal LSM and multiphoton LSM, allows for the visualization of thin planes from within a thick bulk sample, a technique known as optical sectioning. In confocal LSM, signals generated by the sample outside of the optical focus are physically blocked by an aperture, preventing their 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 Wide Field Epi-Fluorescence**

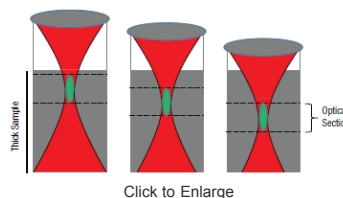


**Figure 2 Optical Sections (Visualization of Thin Planes within a Bulk Sample)**

#### Optical Sectioning in Confocal Microscopy



#### Optical Sectioning in Multiphoton Microscopy



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.

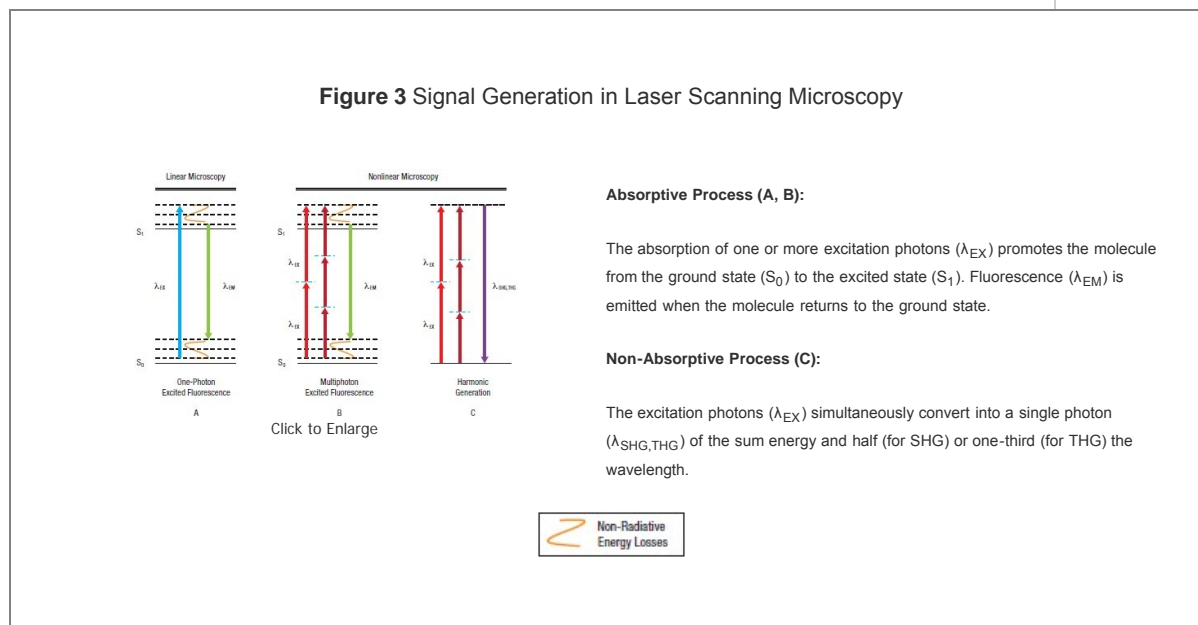
Further constituent discrimination can be obtained by observing the physical order of the harmonic generation. In the case of second harmonic generation (SHG), signal is only generated in constituents that are highly ordered and lacking inversion symmetry. Third harmonic generation (THG) is observed 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.

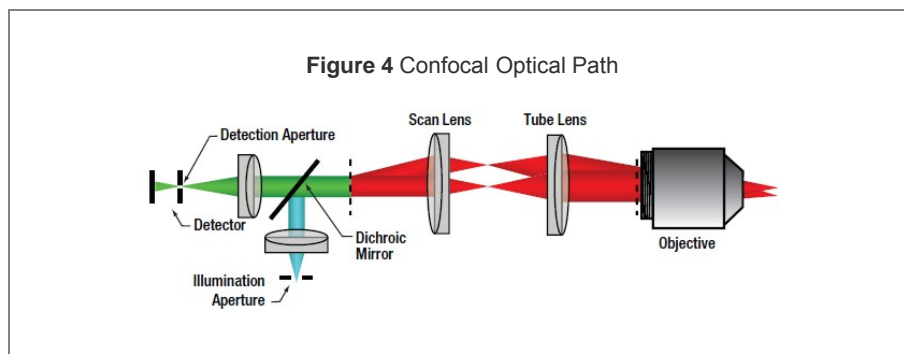


## Image Formation

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.



## 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 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 ( $\lambda_{EX}$ ) and numerical aperture (NA) of the objective lens, as seen in Equation 1.

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

Equation 1 Spot Size

In actuality, the beam isn't focused to a true point, but rather to a bullseye-like shape called an Airy disk. The spot size is the distance between the first 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 determined by the distance required to observe two points as two distinct entities. According to the Rayleigh criterion, these two points are said to be resolvable when the maximum of one point falls no closer than the first zero of the Airy pattern of the adjacent point—that is, when the center of one bullseye falls no closer than the middle of the first ring around the center of the second bullseye. Lateral resolution ( $R_{\text{lateral}}$ ) is therefore:

$$R_{\text{lateral}} = \frac{1}{2} \text{AU} = \frac{0.61\lambda_{EX}}{NA}$$

Equation 2 Lateral Resolution, Confocal LSM

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. The axial resolution ( $R_{\text{axial}}$ ) of a confocal microscope is given as:

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

Equation 3 Axial Resolution, Confocal LSM

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

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

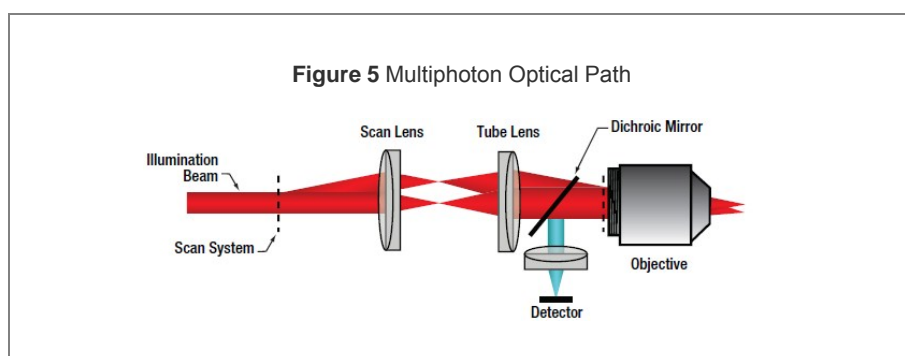
$$D_{\text{pinhole}} = M_{\text{objective}} \times M_{\text{scan head}} \times \text{Spot Size}$$

Equation 4 Pinhole Diameter

As an example, the appropriate size pinhole for a 60X objective with  $NA = 1.0$  for  $\lambda_{EX} = 488 \text{ nm}$  ( $M_{\text{scan head}} = 1.07$  for the Thorlabs Confocal Scan Head) would be  $38.2 \mu\text{m}$  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  $\mu\text{m}$  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.



### 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.

The longer wavelength used for excitation would lead one to believe (from Equation 2 above) that the resolution in nonlinear microscopy would be reduced 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. The lateral resolution of a nonlinear microscope is:

$$R_{\text{lateral}} = 2 \ln(2) \frac{0.325\lambda}{\sqrt{2}(NA)^{0.91}}$$

Equation 5 Lateral Resolution, Multiphoton LSM

and the axial resolution is:

$$R_{\text{axial}} = \frac{0.532\lambda}{\sqrt{2}} \left( \frac{1}{n - \sqrt{n^2 - (NA)^2}} \right)$$

Equation 6 Axial Resolution, Multiphoton LSM

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 the Rayleigh criterion (Equation 2) 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 297 nm (Equation 2) and the pixel size in the displayed image should be  $\sim 129$  nm. Therefore, for a 1024 x 1024 pixel capture resolution, the scan field on the specimen would be  $\sim 132 \mu\text{m} \times 132 \mu\text{m}$ . 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 ( $I^{-4}$ ) of scattering on wavelength. Typical penetration depths for multiphoton LSM are 250 - 500  $\mu\text{m}$ , although imaging as deep as 1 mm has been reported in the literature, compared to  $\sim 100 \mu\text{m}$  for confocal LSM.

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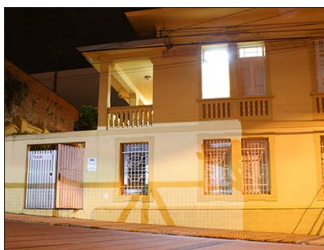
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