

Multiphoton Excitation Microscopy

MPE Tutorial



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Introduction

Multiphoton excitation (MPE) microscopy is a powerful tool that combines scanning microscopy with multiphoton fluorescence to create high-resolution, three-dimensional images of microscopic samples. MPE is particularly useful in biology because it can be used to probe delicate living cells and tissues without damaging the sample. Although multiphoton excitation has been demonstrated with high-power cw argon and krypton lasers, the laser source of choice for MPE microscopy is an ultrafast Ti:Sapphire laser.

Advantages of Multiphoton Excitation Microscopy

When compared to conventional confocal microscopy, MPE microscopy has many advantages:

- higher axial resolution
- greater sample penetration
- reduced photobleaching of marker dyes
- increased cell viability

Organization of This Tutorial

The first section of this tutorial, *Theory*, will discuss the basic theory and concepts of multiphoton fluorescence and confocal microscopy. These two concepts will then be brought together in a discussion of MPE.

In the second section, *Experimental Set-ups*, the equipment needed for a typical application will be described, along with useful information on procedures and protocols.

The third section, *Glossary*, will provide definitions and descriptions of words and concepts common to MPE experiments.

Multiphoton excitation microscopy is an amalgamation of multiphoton fluorescence and confocal scanning microscopy. To fully understand MPE microscopy, it is important to have a basic understanding of these two techniques.

Multiphoton Fluorescence

In traditional fluorescence spectroscopy, a single photon of light is used to excite a molecule from its ground state (S_0) to an upper energy state ($S_{1(n)}$), as shown in *Figure 1*. Once excited, the molecule then decays to an intermediate energy state ($S_{0(n)}$), giving off a photon of light (fluorescence) that is representative of the difference in energy between those states. The relationships between photon energy (E) frequency (ν), and wavelength (λ) are given by the equations:

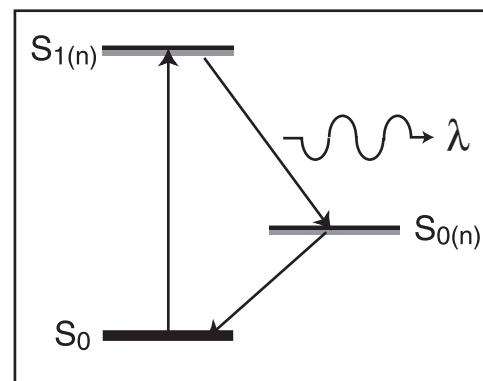


Figure 1. Simplified three-level energy diagram.

Photon Formulae

$$E = h\nu$$

$$\nu\lambda = c$$

$$\lambda = hc/E,$$

where

λ = wavelength

ν = frequency

E = energy

C = speed of light

h = Planck's constant

$$E = h\nu, \nu\lambda = c, \text{ and } \lambda = hc/E,$$

where h is Planck's constant and c is the speed of light. Since the energy difference between the ground state and the upper energy state ($S_{1(n)} - S_0$) is greater than the energy difference between the upper state and the intermediate state ($S_{1(n)} - S_{0(n)}$), it is evident from these equations that the energy of the exciting photon is greater than that of the fluorescing photon, and thus, the wavelength of the exciting photon must be shorter than that of the fluorescing photon.

Multiphoton Interactions

Although the interaction probability is greatest for single-photon absorption, if two or more lower energy (longer wavelength) photons arrive simultaneously, there is some probability that they can excite the molecule as long as

$$(E_1 - E_0) = hc (1/\lambda_1 + 1/\lambda_2 \dots + 1/\lambda_n)$$

where $\lambda_1 \dots \lambda_n$ are the wavelengths of individual photons. This is demonstrated in *Figure 2*, where a 5 eV electronic transition in a serotonin molecule can be excited by a single 250 nm photon (deep ultraviolet), two 500 nm photons (green), or three 750 nm photons (near-infrared).

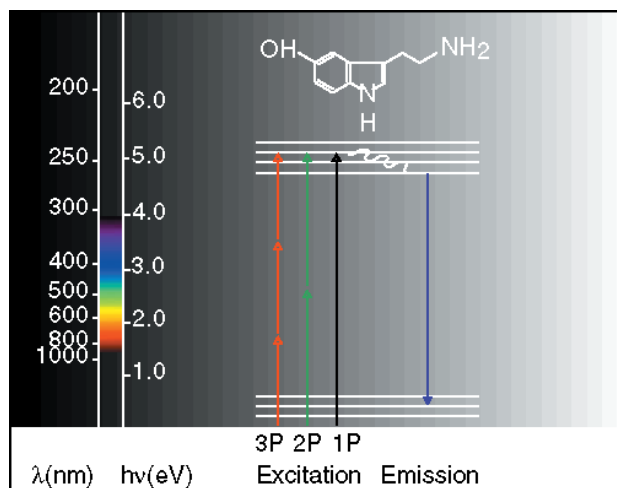


Figure 2. Multiphoton absorption in a serotonin molecule.

Probability for Absorption

Single-photon:	$\propto I$
Two-photon:	$\propto I^2$
Three-photon:	$\propto I^3$

The probability of two-photon absorption is much smaller than that for single-photon absorption, and the probability of three-photon absorption is smaller still. The absorption probability, however, is nonlinear and increases with the square of photon intensity (I^2) for two-photon absorption and as the cube of photon intensity for three-photon absorption. Since intensity is a measure of power per unit area, the high peak power and focusability of ultrafast pulses mean that modelocked Ti:Sapphire lasers like the Mira[®] and Vitesse[™] are ideal sources for multiphoton applications.

Although two-photon fluorescence using a cw laser is possible, excitation by a Gaussian pulse with a pulsewidth (τ) of 200 fsec ($1.0 \text{ fs} = 1.0 \times 10^{-15} \text{ sec}$) and a pulse frequency (f) of 80 MHz increases the two-photon absorption rate by a factor of 0.56 ($1/\tau f$), or 35,000! Using this relationship, it follows that to achieve the same two-photon absorption rate as a femtosecond laser with an average power of 3 mW to 10 mW, it requires 500 mW to 1800 mW of cw excitation¹ — power levels that could cause extensive photodamage. Another source states that single-mode cw excitation requires 10^2 - 10^3 times more average power than pulsed excitation to yield the same rate of two-photon excitation.² In fact, according to Winfried Denk, who co-invented multiphoton microscopy, “The use of such short pulses and small duty cycles is, in fact, essential to permit image acquisition in a reasonable time while using ‘biologically tolerable’ power levels.”³³

¹ S.W. Hell, M. Booth, S. Wilms, C.M. Schnetter, A. K. Kirsch, D.J. Ardnt-Jovin, and T.M. Jovin, “Two-photon near- and far-field fluorescence microscopy with continuous-wave excitation,” *Opt. Lett.*, 23, 1238-1240 (1996).

² C. Xu and W. W. Webb, “Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm,” *J. Opt. Soc. Am. B.*, 13, 481-491 (1996).

³ W. Denk, D.W. Piston, and W.W. Webb, in *Handbook of Biological Confocal Microscopy*, 2nd ed., edited by James B. Pawley (Plenum Press, New York, NY, 1972), Chap. 28, pp. 445-450.

Rayleigh Scattering

$$\propto 1/\lambda^4$$

Scattering produced by small particles is proportional to the inverse fourth power of the wavelength of light being scattered. Thus the longer wavelengths used for multiphoton excitation will be scattered much less by small particles than the visible wavelengths used for conventional confocal microscopy.

Localized Fluorescence

Another advantage of multiphoton absorption is illustrated in *Figure 3*. With single-photon absorption, when a laser is focused to a point within a sample, the sample may, because of the large probability of single-photon absorption, fluoresce throughout the entire beam path. Using multiphoton absorption, induced fluorescence occurs only at, or near, the focal point of the beam. Since the position of the focal point can be precisely determined, multiphoton fluorescence can yield a great deal of information about specific points below the sample surface. Furthermore, longer wavelengths, particularly the near-infrared, penetrate deeper in biological materials and are not scattered as much as shorter wavelengths.

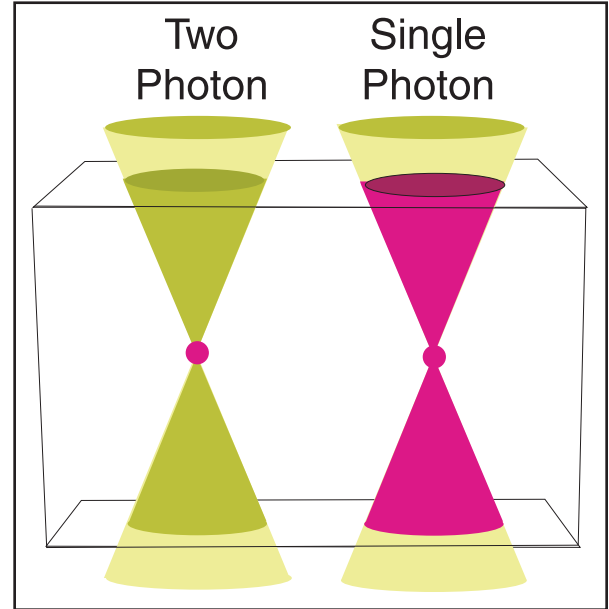


Figure 3. Pink volume illustrates two-photon and single-photon fluorescence induced by a focused laser beam⁴.

⁴ Modified image from: Center for Biomedical Imaging Technology, "Two-photon microscopy," http://www.cbit.uchc.edu/microscopy/two_photon.html.

Laser Scanning Confocal Microscopy

Confocal Microscopy

Advantages

- Lower-cost probe laser
- Higher lateral resolution

Disadvantages

- Lower contrast
 - Limited depth penetration
 - Photodamage
-
-

Laser scanning confocal microscopy (LSCM, also referred to as CSLM, confocal scanning laser microscopy) has been established as a valuable tool for obtaining high-resolution images and three-dimensional reconstructions of a variety of biological specimens.

The basic operation of a confocal laser microscope is shown in *Figure 4*. A beam of laser light (usually from an argon or krypton ion laser) is focused onto a fluorescent specimen by a microscope objective lens. The fluorescent energy from the sample is then collected through the same

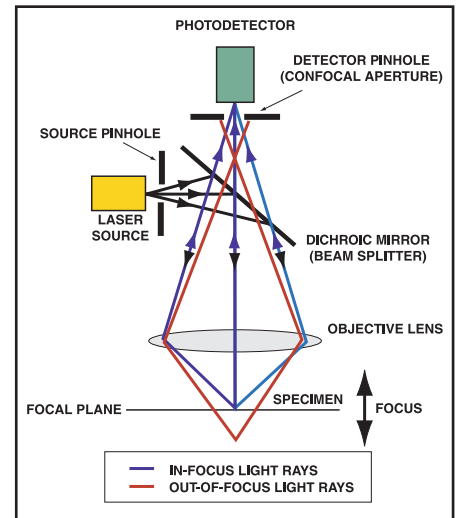


Figure 4. Simplified optics of a confocal laser microscope⁵.

⁵ Lance Ladic, "Simplified optics of a LSCM," <http://www.cs.ubc.ca/spider/ladic/images/optics.gif>.

microscope objective and recorded by a photodetector. The optical system is designed so that the laser's focal point in the sample is imaged exactly on the face of the photodetector (i.e., confocal). By its nature then, any fluorescence emanating from the point of laser focus will be focused on the photodetector, and any fluorescence emanating from *points other than the point of laser focus* will be out of focus at the photodetector. Thus, by inserting a small aperture in front of the photodetector, the gathered fluorescence can be limited to a region very close to the point of focus of the laser. The smaller the aperture is, the higher the resolution will be.

In LCSM, the focal point of the laser spot is stepped across the sample in a raster (x-y) pattern, always maintaining the confocal nature of the image at the detector. Fluorescence information is accumulated on a point-by-point basis with a digital processing system, and a fluorescent cross section of the sample at the focal plane is obtained. By stepping the focus vertically (z), multiple slices can be used to build up a full three-dimensional image. With non-opaque samples, the interior structure can be clearly seen. By scanning in the x-z direction, a vertical cross section can be obtained.

Problems with LCSM

When working with biological samples, serious problems can occur with normal confocal fluorescence microscopy. One problem is photobleaching of the fluorescent label (fluorophore). In many cases, researchers are interested in observing living specimens, often at several stages during development. Because the small confocal aperture blocks most of the light emitted by the tissue, including light coming from the plane of focus, the exciting laser must be very bright to allow an adequate signal-to-noise ratio. This bright light causes fluorescent dyes to fade within minutes of continuous scanning. Thus the fluorescence signal weakens as subsequent scans are made, either to produce a three-dimensional image or to observe a single slice at several time points. Phototoxicity is another problem. Many fluorescent dye molecules generate cytotoxins like singlet oxygen or free radicals, and one must limit the scanning time or light intensity to keep the specimen alive.

Multiphoton Excitation Microscopy

MPE Microscopy

Advantages

- High contrast
- No bulk fluorescence
- All fluorescence from focus point is collected by detector
- Less damage to living tissue
- Increased penetration depth

Multiphoton microscopy solves the problems of LSCM: improving the signal-to-noise ratio by eliminating fluorescence except at the focal point of the laser, and reducing or eliminating photobleaching and phototoxicity by using *low average power*.

There are two main differences between multiphoton and confocal microscopy:

- The source is an ultrafast laser (usually Ti:Sapphire) with very high peak power but low average power.
- The confocal aperture is unnecessary, because all of the fluorescent light originates from the laser focus spot.

The differences between multiphoton microscopy and confocal microscopy are shown in *Figure 5*⁶.

Alternate Detector Configurations

Because TPLSM requires neither an aperture nor focused light at the detector, the emitted light does not have to pass through the microscope at all. For example, a photodetector could be placed on the far side of the sample.

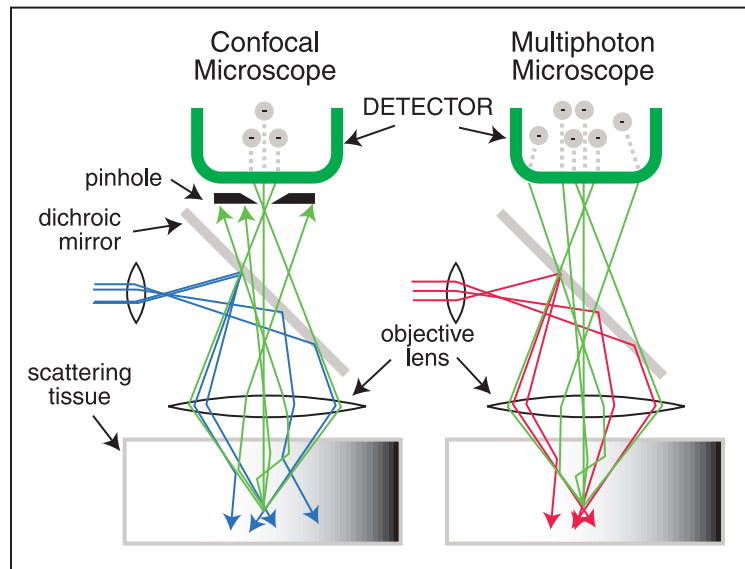


Figure 5. Comparison of a confocal and multiphoton microscope.

In the confocal case, fluorescence occurs throughout the sample and must be blocked by the pinhole aperture. This not only eliminates the fluorescence away from the focal point, but also the scattered (diffusing) fluorescence from the focal point. Only the ballistic (straight line) fluorescence is detected. In the multiphoton case, both the ballistic and the diffusing photons are collected. Furthermore, since the excitation wavelength has a longer wavelength, less excitation light is lost to scattering.

⁶ Microcosm, Inc., "Multiphoton fluorescence microscopy," <http://www.microcosm.com/tutorial/tutorial3.html>.

Enhancing Axial Resolution with Three-photon Microscopy

As was mentioned above, an important benefit of multiphoton microscopy is the improved axial resolution brought about by the nonlinear processes involved. In two-photon processes, the excitation cross section is proportional to the square of the laser intensity. Furthermore, the intensity of a Gaussian beam decreases roughly as the square of the distance from the peak. Consequently, the cross section for two-photon fluorescence is inversely proportional to the *fourth power* of the distance from the focal point of the laser beam.

Use of three-photon excitation can enhance the z-axis resolution even more, as demonstrated in *Figure 6*. In this example, the focal point of an ultrafast Ti:Sapphire laser was moved from a cover glass into a fluorescing film (the laser was operating at 900 nm). In curve *a*, an ultraviolet transition (300 nm) in BBO/toluene was probed by three-photon excitation. In curve *b*, a blue transition (450 nm) in rhodamine 6G was probed by two-photon excitation. The smaller cross section and greater nonlinearity of the 3-photon transition significantly increases the z-axis resolution⁷.

Resolution

In general, surface (x-y) resolution with TPLSM microscopy is slightly worse than with LSCM using the same fluorophore, because the excitation wavelength is twice as long. The real benefits are in axial resolution, and in the ability to penetrate more than twice as deep into biological samples without damaging the sample.

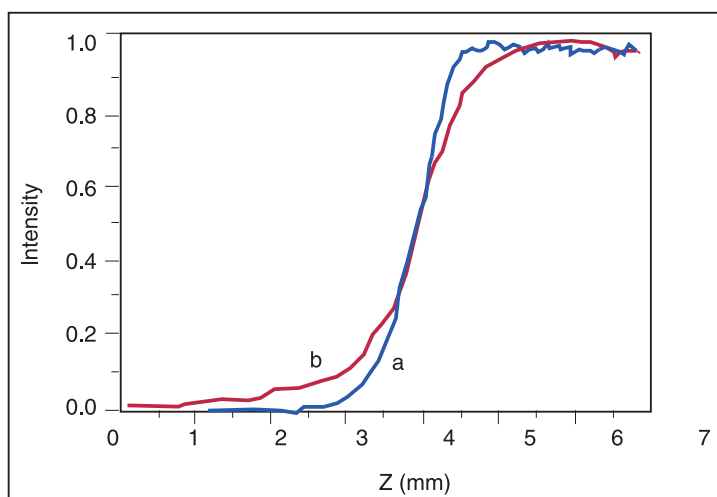


Figure 6. Resolution along the z-axis for two-photon and three-photon excitation.

⁷ Microcosm, Inc., "Multiphoton fluorescence microscopy."

Experimental Set-up

The schematic of a typical multiphoton excitation microscope setup is shown in *Figure 7*. The lower portion of the microscope uses a conventional optical microscope objective. The upper portion includes a photomultiplier tube (or other photon detector) that is filtered to eliminate stray light from the laser or other source; a dichroic mirror that reflects the near-infrared laser light down through the objective, while transmitting the visible fluorescent light to the photodetector, and to an x-y raster scanning unit that can rapidly deflect the laser beam over the objective field.

Control electronics synchronize both the x-y raster scan and the detector with pulses from the modelocked laser. Microcomputers and workstations are used to store and process the data, and to create three-dimensional images⁸.

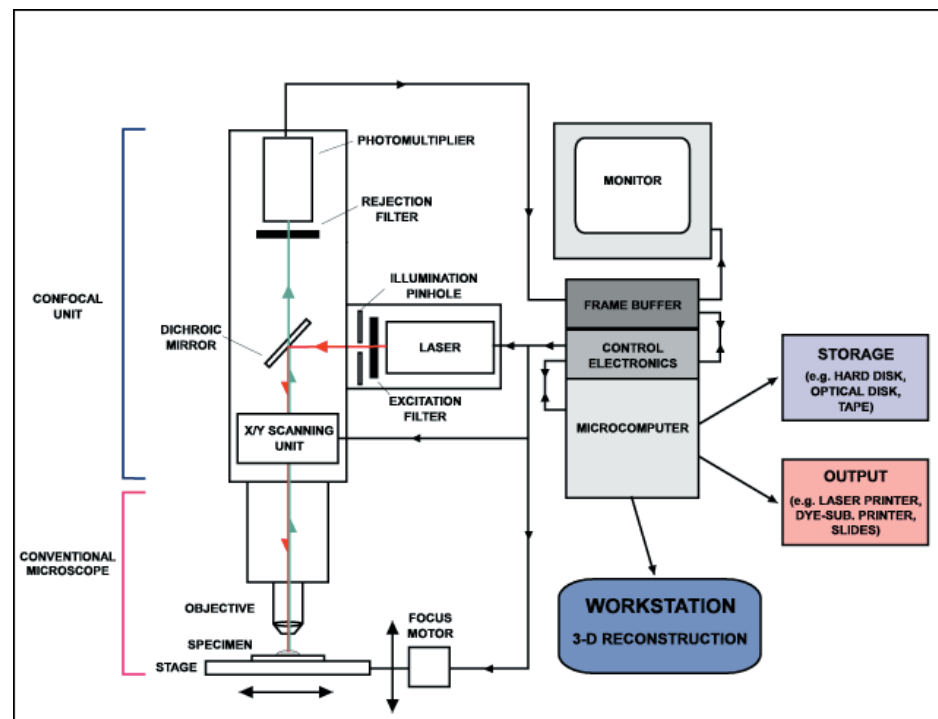


Figure 7. A typical multiphoton microscope set-up.

⁸ Lance Ladic, "A typical LSCM system," <http://www.cs.ubc.ca/spider/ladic/images/system.gif>.

An Actual Set-up

Figure 8 shows an actual two-photon laser scanning microscope in use at the biology department of the California Institute of Technology. The microscope is a modified Molecular Dynamics Sarastro 2000 confocal scanning unit used with a Nikon Optiphot 2 upright microscope. Only minor modifications were made to allow two-photon imaging, and the ability to do standard confocal imaging (i.e., by reinserting the confocal pinhole) has been retained. Two-photon imaging is carried out using a Coherent Mira 900 modelocked Ti:Sapphire laser, pumped by a Coherent Innova® 310 8W argon-ion laser⁹.

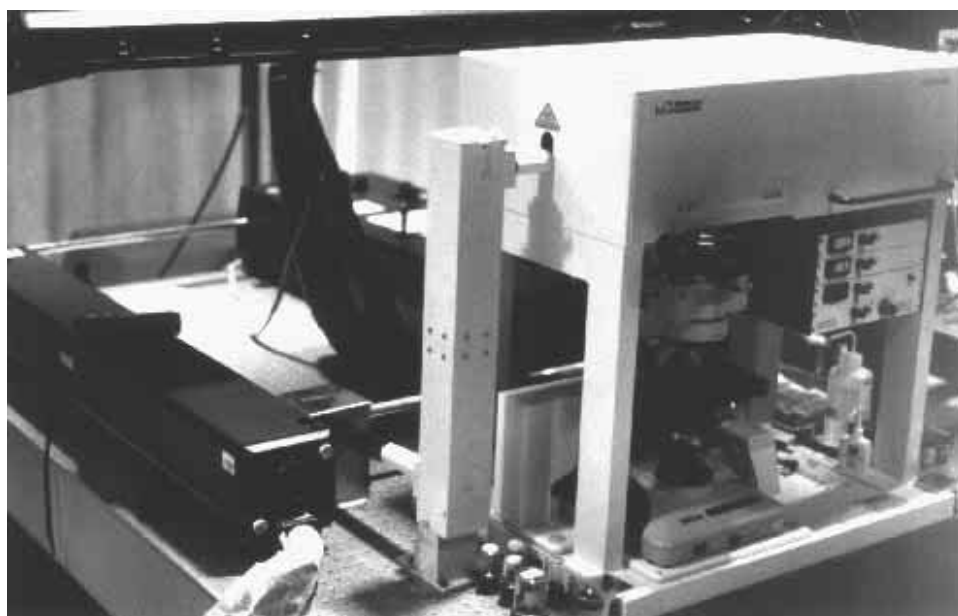


Figure 8. The CalTech two-photon scanning laser microscope.

The two periscope mirrors that bring the laser beam up to the optical table of the Sarastro 2000, as well as the two mirrors that bounce the beam from the Mira 900 to the periscope, are optimized to reflect infrared. All of the laser beams are enclosed in metal tubes and boxes to allow safe operation.

Molecular Dynamics ImageSpace software controls the Sarastro 2000 and does all the data acquisition, image processing, data manipulation and data quantification.

⁹ Steve Potter, "Two-photon laser-scanning microscope," <http://broccoli.caltech.edu/~pinelab/2PhEquip.html>.

Critical Equipment — Microscope and Laser

Although there are many components in an MPE microscope, including the data acquisition software and hardware, the most critical components to the success of the system are the microscope and the ultrafast laser.

MPE Microscopes

Most existing laboratory systems have been built by modifying an existing confocal scanning microscope (essentially removing the confocal aperture) and attaching an ultrafast laser system. Now, several manufacturers, including Zeiss, BioRad, and Leica, are offering microscopes specifically designed for MPE applications.

Ultrafast Laser Systems

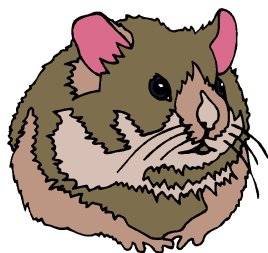
Coherent offers several ultrafast laser options, including fixed-frequency turnkey lasers (the BioLight™-1000 and the Vitesse) and tunable sources (the Vitesse-XT and the Mira Optima™).

The BioLight-1000 is designed specifically for MPE applications where cell viability is critical. This compact, diode-pumped, solid-state, modelocked Nd:YLF laser produces 1047 nm light, and studies have shown that cell viability increases dramatically at wavelengths above one micron (see sidebar).

The Vitesse and the Vitesse-XT are turnkey systems that combine our Verdi® (DPSS) pump source and a Ti:Sapphire femtosecond laser in a compact, fully integrated package. The standard Vitesse operates at a fixed wavelength. The Vitesse-XT includes fully computer-controlled wavelength tunability and is ideal for many MPE applications. Both units provide hands-off operation.

For the majority of MPE set-ups the most important requirement is versatility. For customers who need the highest level of flexibility and control, a Mira/Verdi combination offers the best solution. Mira Optima 900 modelocked Ti:Sapphire lasers offer several advantages in scientific research environments. X-Wave™ optics are standard on all the Mira models, making the system tunable over the entire Ti:Sapphire range (700 nm to 1000 nm). Optima, an onboard diagnostic and control system, makes laser alignment simple and routine.

It Lives!



At the University of Wisconsin—Madison, researchers used MPE to image cell division in hamster embryo. With confocal imaging at approximately 520 nm, cell division stopped. With MPE imaging at 1047 nm, cell division continued unabated. The embryo was then implanted in a female hamster and brought to term. The result—a healthy female hamster who now has a litter of her own.¹⁰

¹⁰ Randall C. Willis, "Examining live embryos nondestructively," *Biophotonics Int.*, Nov/Dec 1999, pp. 42-44.

Because of their simplicity and hands-off operation, the BioLight-1000 and Vitesse family of lasers are particularly suited for biology laboratories where the researchers have not had extensive laser experience. In this case, the benefits of hands-free control will outweigh any constraints due to wavelength limitations. However, the Mira Optima 900 is still the industry standard for ultrafast lasers. The Mira can be used with a variety of pump sources and, when combined with Mira accessories, this system provides tunable ultrafast performance from the UV to the mid-IR. The ultimate choice of laser will depend on both the specific experimental requirements and a customer's needs.

Attaching the Laser and Microscope

Ultrafast lasers are large, and cannot be attached directly onto the microscope. Two methods are available to attach the laser and microscope: direct and fiber coupling. Directly coupling the laser beam into the microscope is accomplished with relay mirrors. Fiber coupling uses a fiber-optic waveguide to guide the beam into the scanhead. Both methods have advantages and disadvantages.

Direct coupling (used in the CalTech system shown earlier) provides a simple solution for delivery of ultrafast pulses at a higher average power than is possible with a fiber delivery set-up. However, maintaining alignment of the relay mirrors can be a problem, and the beam path should be enclosed to prevent accidentally exposing the operator to the beam. In addition, group velocity dispersion from the microscope optics can result in a broadened pulsewidth, which will effect both the two-photon absorption and the imaging quality.

Fiber coupling eliminates enclosure issues, but requires a grating compensation system that enables fiber delivery of ultrafast pulses without risk of self-phase modulation. This grating compensation allows the user to vary the pulsewidth and dispersion characteristics of the pulse in order to compensate for group velocity dispersion in the microscope. It also allows the laser to be vibrationally isolated from the microscope, and helps facilitate scanhead alignment in set-ups using multiple microscopes. There are, however, several constraints with fiber delivery. The necessity of delivering an ultrafast pulse without self-phase modulation puts an upper limit on the power delivered by the fiber. This results in lower average power when compared to directly coupled systems. Fiber delivery also can cause limitations in the tuning range due to constraints in fiber design.

Recommended Accessories and Diagnostic Equipment

When dealing with any ultrafast laser experiment, it is important to have a stable environment and to have the ancillary equipment necessary to measure and verify laser parameters. Extremely small variations in laser cavity spacing and alignment can have a major effect on the output of the laser system. Coherent's ultrafast laser systems, like the Mira and Vitesse, include internal diagnostics and correction systems to automatically ensure proper performance under most laboratory conditions. Nevertheless, independent monitoring of output characteristics ensures optimum performance.

Vibration Isolation

Ultrafast experiments require a relatively vibration-free environment, and the laser should always be mounted on a vibration-isolation table. For best results we recommend well-damped, 8-inch-thick tables mounted on air supports. Coherent can supply these tables to meet your site-specific or unique application needs.

Beam Diagnostic Instrumentation

The key optical parameters in an ultrafast laser system are wavelength, average power, peak power, pulsewidth, and pulse repetition rate. In addition, it is important to know that the laser is fully modelocked, and to be alerted to cw breakthrough.

Measuring Wavelength

The Mira Optima 900 comes with the micrometer settings calibrated. These settings will generally be good to about ± 1 nm, so in many cases where the customer is exciting a sample with a broad absorption cross section (for example, MPE), this level of accuracy will be sufficient — especially since the bandwidth of the pulse is 5 nm to 10 nm.

For customers who need to know the center wavelength more accurately, a WaveMate™ is a fine solution. The WaveMate, an inexpensive wavemeter manufactured by Coherent, measures wavelength with 0.1 nm accuracy and is ideal for most applications.

Measuring Pulsewidth

The width of an ultrafast laser pulse cannot be measured directly. Instead, the pulsewidth must be inferred from secondary measurements. The preferred method for determining pulsewidth is to use an *autocorrelator*. An autocorrelator determines the pulsewidth by separating the beam into two parts and focusing them to the same point in an angle-matched nonlinear crystal; then observing the second-harmonic output while varying the path-length difference between the two beams. By making assumptions about the shape of the pulse (e.g., Gaussian, Lorentzian), the output is de-convoluted and the pulsewidth obtained.

Autocorrelators are available from several sources, for example, APE in Berlin, Germany. All models provide pulsewidth data, and some models also provide wavelength information. The main drawback of these devices is their cost.

A less expensive alternative is a commercial (Rees) spectrometer with additional computer software provided by Coherent. In this case, the bandwidth of the pulse can be displayed on a standard personal computer. The pulsewidth is approximated, based on the bandwidth. These devices are less accurate than an autocorrelator ($\sim\pm 10\%$) but are fine for MPE applications. They also provide wavelength data and can be used to monitor for cw breakthrough.

Measuring Average Power

Coherent offers a variety of power and energy meters suitable for measuring the average output power of an ultrafast system. Coherent's LaserMate™ and LabMaster™ power meters, with appropriate detectors, are particularly well-suited.

Measuring Peak Power

Unfortunately, conventional power and energy meters cannot measure the peak powers of ultrafast systems directly, because the pulse repetition rate (~ 80 MHz) and the pulsewidth (< 100 fsec) are beyond the bandwidth and resolution limits of the instruments. Consequently, the peak power must be determined by first determining the pulsewidth and repetition rate of the system, and then calculating the peak power by the formula

$$P_{\text{peak}} = P_{\text{avg}} / (f \times \tau)$$

where f is the pulse repetition rate and τ is the pulsewidth. The pulse repetition rate is fixed by the laser geometry, which can be found in the laser specification table. The pulsewidth is best determined by using an autocorrelator.

Glossary

Autofluorescence

Some biological samples contain naturally occurring fluorophores (e.g., serotonin, NADH, flavins) that can be used as marker tags in fluorescence microscopy, without the introduction of additional dyes.

Brightness

The intensity per unit area of a beam projected onto a plane normal to the direction of propagation. Brightness is also known as luminance and luminous sterance.

Confocal aperture

In confocal microscopy, the limiting aperture is placed in front of a detector at the focal point of the imaging system. Its purpose is to eliminate all light emanating from points other than the focal point of the laser.

Contrast

Contrast is the luminance of an image or point of interest with respect to the background (or other points that are not of interest). Contrast is defined as $(L_i - L_b)/L_b$, where L_i is the luminance of the image, and L_b is the luminance of the background. Because luminescence away from the point of interest is dramatically reduced or eliminated in MPE microscopy, MPE images generally have higher contrast than confocal microscope images.

CW breakthrough

In a modelocked laser, if any part of the system goes out of alignment or synchronism, an unwanted continuous-wave (cw) component in the output beam can seriously degrade an MPE experiment by causing unwanted bulk fluorescence and photobleaching, thermal damage, reduced peak pulse power, increased pulsewidth, and other undesirable effects. In the Vitesse-XT and Mira Optima systems, output is monitored. If these systems detect cw radiation, they automatically send a signal to the starter to re-initiate modelocking.

Fluorophores

Fluorophores are fluorescent dyes that can be introduced into a sample and attach themselves to features of interest. Some fluorophores suited for multiphoton excitation, along with their two-photon excitation wavelength, are shown in the table below. Two-photon absorption cross sections are quite broad, and the optimum excitation wavelength depends on the solvents, pulsewidth, laser power and other factors.

The vast majority of current MPE applications are related to calcium (Ca^{2+}) imaging (700 nm to 720 nm excitation), "wild-type" green fluorescent protein (GFP) imaging (800 nm to 850 nm excitation), and enhanced GFP imaging using a mutated protein with an order of magnitude greater fluorescence (900 nm to 950 nm). Other fluorophores are listed in *Table 1*, which follows.

Fluorophore	Wavelength (nm)	Fluorophore	Wavelength (nm)
Bis MSB	690	Dil	700
Bodipy	920	Fluorescein	780
Cascade Blue	750	Indo-1	700
Coumarin 307	775	Lucifer Yellow	860
DAPI	700	Rhodamine B	840
Dansyl Hydrazine	700		

Table 1. Two-photon absorption wavelengths.

Intensity

The power (flux) per unit solid angle of a laser beam.

Kerr lens effect

When an optical medium is placed in a strong electrical field, the index of refraction changes. This is known as the Kerr effect. Light is an electromagnetic wave. When a focused Gaussian laser beam passes through a Ti:Sapphire crystal, the electric field generated by the beam causes a nonhomogeneous change in the index of refraction, creating a weak lens that, along with the geometry of the laser cavity, results in higher gain for modelocked pulses than for cw pulses.

Modelocking

The ability to generate a train of very short pulses by modulating the gain or excitation of a laser at a frequency with a period equal to the round-trip time of a photon in the laser cavity (frequency = $c/2nL$). The resulting pulsewidth depends upon the gain bandwidth of the laser medium (the larger the bandwidth, the narrower the pulse), the accuracy of the frequency setting, and the stability of the laser cavity. Ti:Sapphire lasers like the Mira and Vitesse are self-modelocked using the *Kerr Lens Effect* to generate modelocked pulses with output pulsewidths in the 50 fs to 150 fs regime.

Optical sectioning

The ability to obtain an image of a planar layer of a sample at various points within the sample. A section can be either horizontal (x-y) or vertical (x-z), or a combination thereof. Optical sectioning is a major strength of scanning MPE microscopy, due to its ability to penetrate deeper into a sample, and the enhanced contrast brought about by fluorescing only at the focal point of the laser probe.

Photodamage

Damage to a sample caused by exposing it to intense light. Damage can be caused by heat, ablation, bleaching, or the creation of singlet oxygen. For most biological samples, infrared light is less destructive than visible or ultraviolet light. Using a low-duty-cycle modelocked laser can minimize or eliminate heat damage.

Pulsed laser parameters

The following terms are commonly used to specify the performance of pulsed lasers. (The equation for peak power depends upon the shape of the pulse, and is exact only for square pulses.) The values given are for a 10 mW average power laser operating at a 100 MHz pulse rate with a 100 fsec pulsewidth.

Energy per pulse = average power/pulse rate = 0.1 nJ

Peak power = energy per pulse/pulsewidth = 1000W

Duty cycle = pulsewidth x pulse rate = 0.001%

Period (time between pulses) = 1/pulse rate = 100 μ sec

Pulsewidth limit

The uncertainty principle demands that the product of the pulsewidth and the spectral bandwidth of a laser pulse have a lower bound (i.e., $\Delta\lambda \times \Delta\tau \geq \text{Constant}$). Consequently, as the pulsewidth decreases, the spectral bandwidth increases. For example, a 70 fsec pulse is spread over 13 nm¹¹. Group velocity dispersion (GVD) in the microscope optics also increases as the pulsewidth decreases, further broadening the pulse. For two-photon and three-photon excitation, the absorption bandwidths typically correspond to the spectral bandwidths obtained from 50 to 100 fs pulse. Decreasing the pulsewidth beyond these limits results in less efficient absorption¹².

Resolution

The lateral resolution of a single-photon fluorescence microscope versus a multiphoton fluorescence microscope depends strongly on whether the excitation wavelength or the fluorophore remains constant during the comparison. If the wavelength remains constant, the resolution is the same for both systems. If the fluorophore is the same (meaning that the excitation wavelength is doubled or tripled in the multiphoton case), the resolution of the multiphoton system can be degraded by as much as a factor of two.

Uncaging experiments

In certain biological samples, ions (typically Ca²⁺) are trapped (caged) within the cellular structure. By exciting these ions with ultraviolet light (<400 nm), they can be released as free ions (uncaging). Unfortunately, the application of ultraviolet light can damage the surrounding cells. By using multiphoton excitation, this collateral damage can be avoided.

Working with UV-excited fluorophores

UV excitation presents some special challenges for laser microscopy because conventional glass optical elements do not work efficiently in both the ultraviolet and the visible. Furthermore, when working with biological samples, UV excitation can be very destructive to the sample itself. Multiphoton excitation solves these problems because both excitation and fluorescence wavelengths can be handled by conventional glass optics; and, for most biological samples, the near-infrared excitation wavelength does not damage the sample.

¹¹ Denk, et. al., *Handbook of Biological Confocal Microscopy*.

¹² Xu, et. al., and W. W. Webb, "Measurement of two-photon cross sections."