Multiphoton Microscopy Goes Deeper

MRI and Molecular Imaging
Multiphoton imaging has become a widespread and successful technique for high-resolution 3-D imaging of sensitive living samples, thick tissue sections and even of living animals. However, scientists using the technique are always looking to image deeper, even in highly scattering tissues.

In the past few years, the average power delivered by ultrafast pulsed Ti:sapphire lasers, which are most commonly used in this application, has increased. This capability has enabled deeper tissue imaging, but increases in the average power will damage most samples because of the heating effects of high laser power. A new approach, based on dispersion compensation, can allow imaging to occur deeper in the tissue.

Generating fluorescence

The parameters of imaging systems that affect multiphoton imaging efficiency and penetration depth are:
- The amount of average power available from the laser.
- The amount of peak power delivered to the sample with each pulse.
- Transmission efficiency of the laser beam to the sample.
- The efficiency with which the dye is excited by the laser wavelength used.

These parameters can be expressed in the proportionality:
\[
I(\lambda) \propto P_{\text{ave}}(\lambda) \times P_{\text{peak}}(\lambda) \times T(\lambda) \times E(\lambda)
\]

where \(I(\lambda)\) stands for fluorescence intensity due to multiphoton absorption, \(P_{\text{ave}}(\lambda)\) is the average power, \(P_{\text{peak}}(\lambda)\) is the peak power delivered during each pulse, \(\lambda\) is the laser wavelength, \(T(\lambda)\) is the transmission efficiency of the imaging system, and \(E(\lambda)\) is the excitation efficiency of the dye. Each parameter is a function of laser wavelength.

The practical limit of average power that biological samples can withstand has been exceeded by the commercially available Ti:sapphire laser sources used in multiphoton imaging. These lasers can achieve average power of more than 3 W at the 800-nm peak of the gain curve and >600 mW in a tuning range of 690 to 1020 nm. Typical power used for high-resolution laser scanning in multiphoton imaging is between 1 and 5 mW, depending on the focusing power of the objective.

For video rate imaging, where the scanning dwell time for the laser source is significantly reduced, 10 to 20 mW is typical. Although there are significant power

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Reprinted from the January 2007 issue of Biophotonics International © Laurin Publishing Co. Inc.

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**Figure 1.** Using Sellmeier coefficients, the amount of dispersion vs. wavelength for glass in a microscope is shown.
losses through most commercial microscope systems, further attenuation of the Ti:sapphire laser is necessary to limit damage to tissue.

Given the limit of average power that can be applied to a biological sample, only peak power and dye excitation efficiency can be improved to increase fluorescence generation capability. Because the choice of dye is usually determined by the sample and the experiment, increasing the peak power is the only method remaining to enhance the fluorescence signal.

Peak power can be improved by increasing average power, decreasing the laser repetition frequency and delivering shorter pulses to the sample:

\[ P_{\text{peak}} = \frac{P_{\text{ave}}}{\tau \times F} \]

where \( \tau \) is pulse duration and \( F \) represents the repetition frequency. Delivering lower repetition rates compromises the stability of Ti:sapphire laser architectures caused by the very long cavity lengths. Delivering shorter pulses to the sample by countering the pulse-broadening mechanism of the microscope optics (group velocity dispersion) has been used successfully at single wavelengths. There is a significant challenge to making this feasible for wide wavelength tunability; however, recent advances in automation of this technology now make this possible.

**Understanding dispersion**

The main hurdle to overcome in delivering shorter pulses to the sample is group velocity dispersion. This type of dispersion can be described as a delay of shorter wavelengths with respect to longer wavelengths within the ultrafast pulse as they pass through an optical medium. This results in temporally broadened pulses delivered to the sample in most multiphoton imaging systems installed today.

The broadening typically goes uncorrected because the techniques necessary to compensate for it require a sophisticated optical layout and are usually incompatible with hands-free multiphoton microscopy experiments.

Using Sellmeier coefficients, we can graph the actual amount of dispersion vs. wavelength for a certain amount of glass. A typical commercial multiphoton imaging system has many optical components, including a fused silica acousto-optic modulator, tube lens elements and an objective. The dispersion of a commercial imaging system equipped with a 1.4-NA, 63× oil-immersion objective was measured experimentally and found to be nearly equivalent to 320 mm of fused silica (Figure 1).

The resulting pulse duration after group velocity dispersion effects using the formula:

\[ \tau_{\text{sample}} = \tau_0 \sqrt{1 + \frac{4ln2GVD}{\tau_0^2}} \]

where \( GVD \) (expressed in units of fs²) is the group velocity dispersion introduced by microscope optics and \( \tau_0 \) represents the initial pulse width. For initial pulse duration of 100 fs, the resulting pulse du-
DISPERSION COMPENSATION

rations at the sample when using a 1.4-NA, 63× oil objective are 702 fs for 690 nm, 531 fs for 800 nm and 305 fs for 1020 nm (Figure 2).

Beating dispersion

There are well-known techniques for overcoming group velocity dispersion using prisms. Dispersion compensation has been implemented by many groups that use ultrashort lasers for spectroscopic applications in chemistry and physics, as well as a small group of biological researchers with expertise in optical science whose primary focus is to develop improvements to the optical techniques used in imaging. Although spectacular results have been obtained with dispersion compensation, the significant complexity and lack of automation of the designs conceived to date have prevented the vast majority of researchers from implementing this method.

To enable its widespread use, researchers need the following features from dispersion compensation:

• Full automation: All adjustments necessary should be synchronized with wavelength change of the ultrashort laser source.
• Compactness.
• Sufficient group velocity dispersion compensation at all wavelengths.
• Large dynamic range to compensate for all possible objectives and optical systems.
• Excellent beam pointing stability.
• Excellent beam quality with $M^2 < 1.1$, >90 percent roundness and <10 percent astigmatism.
• Properly collimated output beam.

In response to the demands of biologists, Spectra-Physics has introduced the Mai Tai DeepSee, which addresses all of the parameters above. This fully automated device provides from 0 to

![Figure 4. The effect of dispersion compensation on GFP fluorescence measurements is shown.](image)

![Figure 5. Both images of bovine endothelial cells were taken with 800 nm, 1.5 mW at the sample, and the same PMT gain settings. A Mai Tai HP on a commercial multiphoton imaging system equipped with an acousto-optic modulator, a microscope and a 63× oil-immersion objective was used. The image at left was taken without and that at right with dispersion compensation. Courtesy of Holly Aaron, University of California, Berkeley.](image)
−35,000 fs² of negative group velocity dispersion at 690 nm — enough to compensate for an acousto-optic modulator attenuator, a commercial microscope stand, beam delivery, a 1.4-NA, 63× oil immersion objective and more material if necessary.

The tightly folded DeepSee package can be attached to the front of the laser, extending the length of the combined package by only 1 ft (300 mm). Prisms are automatically adjusted for optimum compensation of various wavelengths. Compensation adjustments are synchronized with wavelength tuning of the Mai Tai so that optimally compensated pulses can be delivered while tuning. Compensation adjustments also can be synchronized with the Z-axis steps of the microscope as a 3-D stack is collected, so that any inherent dispersion of the sample can be corrected for as the stack of images is collected.

Excellent beam pointing stability is maintained through careful design of the optical layout and through the use of monolithic mechanical components that do not drift over time. The output beam is conditioned to reproduce the Mai Tai’s beam size, divergence and waist location optimized for multiphoton imaging. Furthermore, outstanding beam quality is preserved with M² <1.1, >90 percent roundness and <10 percent astigmatism.

**More fluorescence**

The increased fluorescence that can be achieved with dispersion compensation can be calculated using:

\[ I = P_{\text{ave}} \times P_{\text{peak}} \times E(\lambda) \]

This equation expresses the relative amount of fluorescence that can be generated when varying the parameters of average power and peak power. For example, the increase in fluorescence for full laser power on the sample with and without dispersion compensation can be seen in Figure 3.

We can model the effect of dispersion compensation when using a specific dye by imposing the excitation profile of the dye:

The impact that the dispersion compensation has on GFP fluorescence can be seen in Figure 4, and its effect on image quality is seen in Figure 5. The tremendous boost in fluorescence for a given average power that is offered by dispersion compensation will allow biologists to image deeper without concern for damaging the sample with too much average power. Long exposure, in vivo time-lapse experiments also will benefit from the possibility of using less average power and keeping samples alive for longer periods. Complete automation and perfect laser beam characteristics will allow the technology to be implemented in multiuser environments.

**Meet the authors**

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