Ultrafast Laser Supports Bio-Imaging Applications

New Laser-based Methods in Microscopy Open the View to the Cellular and Molecular Level

Optimized Laser Tools for Diverse Applications

There is a growing list of non-linear imaging methods utilizing harmonic generation (SHG and THG) in samples, excitation of endogenous fluorescence, CARS (Coherent anti-Stokes Raman Scattering), Multiplex CARS, as well as several other processes. To support these, laser manufacturers have developed a range of different products.

Fortunately, Ti:S has a wide spectral bandwidth (approximately 650–1100 nm) which allows for substantial flexibility in mode-locked oscillator design. Many researchers still use “open-architecture” oscillators (e.g. Coherent Mira), because they offer the ideal combination of stability and flexibility. They are often preferred where the laser is to be used as a shared resource, because their flexible output enables the widest range of experimental conditions. In addition, to wide wavelength tunability, the cavity can be configured for short pulsewidth (< 100 fs) or for narrow bandwidth and long pulsewidth (10 ps). The pulse repetition rate is the inverse of the roundtrip time of the cavity, which, for most commercial oscillators of this type, is nominally 80 MHz.

A range of optional accessories further augment system flexibility. For example, a pulse picker enables the pulse repetition rate at the microscope to be reduced with minimal effect on peak power. This is sometimes used to minimize deterioration of delicate live samples in long duration experiments. Another accessory is the synchronous OPO (optical parametric oscillator). This provides easy tuning across a wide range of wavelengths, including long wavelengths beyond 1 micron which are useful for deep tissue imaging.

Other options are external synchronization controllers which permit an ultrafast oscillator to be synchronized to an external signal or another oscillator (with less than 100 fs timing jitter). Even more cutting edge are pulse shaping/phase correction devices which allow even a novice laser operator to obtain transform limited pulses and/or novel pulse shapes to enable sophisticated techniques such as Multiplex CARS.

Ultrasound Lasers and 3-Dimensional Imaging

Ultrafast (femtosecond or picosecond) laser pulses are produced by mode-locking the output of a continuous wave (CW) laser with wide gain bandwidth. It’s no coincidence that the use of ultrafast lasers in biological imaging dates from the development of Ti:sapphire (Ti:S) based systems that offered an order of magnitude improvement in laser reliability and ease of use, as compared to earlier dye laser systems. These lasers enabled researchers to focus on developing techniques and taking data rather than building, maintaining and optimizing laser systems.

There are now numerous microscopy techniques using ultrafast lasers that provide 3D imaging capabilities by delivering natural Z axis discrimination – eliminating signals from above and below the focal plane of interest. This is possible because even low average power (a few watts) ultrafast laser oscillators have very high peak power – up to a megawatt. Microscopists use this high power to drive non-linear processes such as two-photon excitation of fluorescent dyes. Here, the laser beam is focused into a sample with a high NA objective. The high order dependence of signal strength on laser power ensures that the signal coming from the beam waist is orders of magnitude higher than that generated even just microns out of focus. Images are then built up by scanning the sample or the laser beam.
One-Box Turn-Key Systems

Laser manufacturers have also developed “one-box” systems, in which the pump and oscillator are enclosed in a single head. These are designed to deliver adjustment-free, optimized performance in bio-imaging applications. For example, tunable one-box oscillators (e.g. Coherent Chameleon) have enabled biologists with limited laser experience to use MPE as a turnkey tool. A key aspect of these lasers is their wide wavelength tuning range (680 nm to 1090 nm) which enables excitation of new fluorophores such as mCherry and mBanana. Also, with any MPE microscope, the final pulsewidth at the specimen is determined by the input pulsewidth and the pulse broadening due to GVD (group velocity dispersion) in the microscope optics. For commercial microscopes with silica optics, the final net pulsewidth is minimized for an initial transform-limited pulse of around 120–140 fs, so these lasers are specifically designed to deliver this pulse duration. This maximizes excitation efficiency and shortens data acquisition times.

Most recently, one-box short pulse oscillators (e.g. Coherent Micra) have been developed to take advantage of the very broad gain bandwidth of Ti:S. Specifically, they utilize novel, broadband optics to deliver pulses with a spectral bandwidth of more than 120 nm. This can be compressed to a transform limited pulsewidth of only 10 fs. Yet this state-of-the-art performance is packaged so that a high level of expertise is not necessary for the operator. These pulses are of interest for bio-imaging for two very different reasons. First, researchers are exploring what happens with MPE and other non-linear imaging techniques when the pulse duration is lowered from over 100 fs to only 10 fs. Does this produce better images, more or less sample damage, etc.? Other researchers are exploiting the broad bandwidth to perform Multiplex CARS covering over 1500 cm⁻¹ of the sample’s Raman spectrum.

To understand how and why these laser systems are used in bio-imaging, it is useful to look at two different examples: the use of a relatively robust technique (THG imaging) to examine key issues in biology, and the development of a new technique (multiplexed CARS) with tremendous potential.

THG Imaging and Embryonic Morphogenesis

Second and third harmonic imaging relies on small (but finite) non-linear coefficients in biological molecules and structures.

These convert small amounts of the focused ultrafast pulse into photons at twice or three times the original frequency (half or one third of the original wavelength). Importantly, both these techniques provide images based on natural optical properties of living systems; transparent, colorless structures can be imaged without the use of stains or dyes which could perturb these systems in unexpected or undesirable ways. SHG requires non centro-symmetric material, but THG can be used to image virtually any optical heterogeneity.

THG is one of several techniques used by the research team of Emmanuel Beaufrepaire at the Laboratoire d’optique et biosciences, in the Ecole Polytechnique (Palaiseau, France). Beaufrepaire explains that, “When an ultrafast beam is focused into a homogeneous sample with a high NA objective, the progressive phase slippage near focus causes destructive interference between third-harmonic radiation created over the focal volume, and completely cancels out the THG emission. But if the beam waist is located at a structural and/or phase interface then axial symmetry is broken by the change in refractive index, and there is a net and observable THG signal. We build up images by a combination of scanning the focused laser spot in XY and Z axes. With a Coherent Mira / APE OPO laser chain tuned to 1200 nm, each image data set is acquired in a total time of between 2 seconds and 2 minutes, depending on whether we’re taking 2D slices or full 3D images.”

In collaboration with a team of biophysicists led by Emmanuel Farge of the Institut Curie (Paris, France), these researchers are using this to study morphogenesis in drosophila (fruit fly) embryos. Morphogenesis is currently a very active research area in biology. It refers to how genes regulate other genes in order to precisely control the development of complex structures in terms of composition, location and size. Beaufrepaire explains, “Biologists know that embryonic development is controlled by complex cascades of gene expression. We wanted to investigate the possible existence of a mechanical component that would provide feedback to this cascade and tell the system when certain growth goals have been achieved. Using THG/2PEF imaging and femtosecond pulse-induced ablation, we
have confirmed the existence of this component and are now investigating details of the mechanisms.”

In this research, the team needed a physical (non-genetic) method to precisely modify developing embryos. They do this by selectively ablating target cells using high energy pulse trains from the laser system. The same laser is then used at a lower power level or at longer wavelengths to take 2PEF or THG images of the embryos as they respond to the damage. Beaurepaire adds that because THG detection is an effective method of imaging physical structures and interfaces, it is a very complementary tool to other techniques that map specific species, for example (See figure 2).

**Multiplex CARS**

Multiplexed CARS was first demonstrated by Michiel Müller and co-workers at the Swammerdam Institute for Life Sciences, University of Amsterdam (Amsterdam, The Netherlands). A team led by Hervé Rigneault at the Institut Fresnel-Mosaic laboratory, CNRS / Paul Cezanne University (Marseille, France) has been developing CARS and Multiplex CARS imaging systems for collaborative studies with biologists, including researchers from the Center of Immunology of Marseille Luminy (Marseille, France).

As its name suggests, CARS imaging relies on producing a CARS molecular signal at the laser beam waist(s). CARS is a 4-wave mixing technique as outlined in Figure 3. In this process a Pump pulse together with a Stokes pulse excite the molecule to a vibrational state that is immediately probed by the Pump pulse to generate the CARS signal at the anti-Stokes frequency. In conventional CARS a molecular vibrational spectrum is built up by scanning the wavelength of the Stokes pulse (the Pump wavelength being fixed) and the frequency difference between the Pump and the Stokes picosecond pulses is tuned to excite a specific molecular Raman resonance. Moving the sample, relative to the laser waist or vice versa, then builds up an image mapping that specific molecular type. Multiplex CARS takes this technique to a whole new level. Here, the Stokes pulse is a broadband pulse and the resultant signal contains all the Raman peaks over a broad (~1500 cm⁻¹) spectral region. The anti-Stokes emission is dispersed in a simple grating spectrometer and detected using a cooled linear CCD array. Each single Multiplex CARS image thus contains information about the concentration and distribution of every Raman active molecule, and is even sensitive to subtle differences in local chemical environment. Rigneault’s team is now in the early stages of using Multiplex CARS to look at model membranes systems. Combined with polarization effects would permit to map the orientation of molecular dipoles in such membranes (see Figure 4).

With current Ti:S laser technology, there are actually several different ways to generate Multiplex CARS images. One method uses a narrowband picosecond oscillator as the Pump and a broadband oscillator as the Stokes. (A narrowband Pump is critical to obtaining well resolved spectral peaks.) Another uses a narrowband Pump and a broadband super-continuum as the Stokes, generated by coupling an ultrafast laser into a photonic crystal fiber. The most elegant method, as used by the Rigneault group, uses just the output of a single broadband laser (Coherent Micra), making optical alignment much simpler than the other methods that use two beams. Specifically, they use a pulse shaper to retard the phase (by π) for a narrow spectral window as proposed by the team of Y. Silberberg at the Weizmann Institute of Science (Rehovot, Israel). The intensity variation due to the weak resonant signal is amplified by heterodyne detection with the strong non-resonant background which acts as a local oscillator.

**Conclusion**

Creative microscopic imaging techniques are allowing key questions in biology to be addressed with a power and precision unimaginable before the use of ultrafast laser sources. The availability of ultrafast lasers optimized to these imaging techniques is enabling their use by an ever broader spectrum of researchers by putting the focus on the science rather than on laser operation and optimization.
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