Fs-Lasers for Life Sciences
Pulsed Laser Applications in Biophotonics

Femtosecond lasers had an unpredictable success in optical sciences ever since they were introduced. Today physicists are still pushing the limits of ultrafast lasers, e.g. the pulse duration down to a single optical cycle and the peak power up to tera or even peta watts. On the other hand, many promising applications have emerged outside the laser laboratories. Mainly the combination of high peak power and low average power make ultrafast lasers very attractive for life sciences. The high peak power enables scientists to take advantage of nonlinear effects while the low average power keeps the cell samples vital/alive. Therefore, it is now possible to gather new information about dynamic life processes that was not accessible before. The studies cover scales reaching from interaction of subcellular compartments up to the physiological reaction of an entire living organism. Some keywords of these applications are “non-invasive”, “high 3D-resolution”, “time-resolved”, “marker-free”, “contact-free” and “high penetration depth” among many others.

High 3D-Resolution Imaging

Most methods in bio-imaging belong either to confocal microscopy or to optical coherence tomography (OCT). The advantage of confocal microscopy is the high resolution imaging (\( \Delta \lambda = 200 \text{ nm} \)) and optical sectioning capabilities (\( \Delta z = 600 \text{ nm} \)) making it possible to virtually reconstruct the whole 3D structure of the sample. However, the involved microscope objectives have high numerical apertures restricting the imaging depth to a few hundred micrometers. On the contrary, OCT allows to image even a depth of a few millimeters inside of tissues but has a somewhat lower axial resolution of a few micrometers. Therefore, confocal microscopy is the technique of choice to study intra- or inter-cellular processes, while OCT is applied if structures in “large” volumes need to be identified, e.g. in ophthalmology.

The major contribution of ultrafast lasers to both fields can be easily understood from the Fourier relation between time and frequency domain: in ultrafast lasers, thousands of resonator modes covering a very broad spectral range are locked together. The coherent nature of the locking mechanism permits the modes to interfere with each other leading to the generation of ultra-short laser pulses. OCT makes use of the coherence in an interferometer-type setup. Here, constructive interference helps to resolve light scattered back at different locations inside the sample. It is a simple fact, that the broader the coherent spectrum, the shorter the laser pulse and the better the axial resolution: a short pulse of a few femtoseconds only can achieve an axial resolution of one micrometer.

Nonlinear Optics and Pulsed Excitation in Microscopy

On the other hand, confocal microscopy benefits from ultrashort laser pulses because of their high peak powers needed for nonlinear optics. Here, nonlinearities yield to many effects: two of them being the generation of new wavelengths starting from the laser line and the sample’s simultaneous absorption of multiple photons. In many cases a single laserline is not enough when dealing with fluorescent molecules which are used as markers in microscopy. Each type of marker has a different absorption peak lying somewhere in the visible spectrum. Several lasers have to be used to cover the whole spectrum and their beams must be overlapped before feeding them into the microscope’s excitation light path. The flexibility to tune the wavelength of a single laser source for the same purpose is therefore useful. Commonly, the desired tunability is achieved with optical parametric oscillators that are synchronously pumped by a femtosecond laser source. More recently, a compact and simpler approach utilizes specially designed fibers. These produce a so-called supercontinuum which either can be tuned and/or spectrally filtered for the desired wavelengths. Besides the wide tuning range also the pulsed excitation of the markers is an advantage: In time correlated single photon counting (TCSPC) the time between the pulsed excitation and the spontaneous emission of a single fluorescence-photon is measured. By building a histogram of the arrival times one can depict the fluorescence lifetime. The latter finally depends strongly on parameters such as the pH-value of the surrounding cytoplasm or the presence of other specific molecules. The marker can therefore be regarded as the smallest possible reporter from inside a cell. Fluorescence lifetime imaging (FLIM) combines the time-resolved

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measurement with the high spatial resolution of a microscope and delivers full 4D information about the sample. Yet another method that was one of the first in the field of “nanoscopy” is based on pulsed excitation: stimulated emission depletion (STED).

Here, two consecutive laser pulses first excite the markers and subsequently deplete their fluorescence again. Only a small fraction of markers which were left out from the depletion-pulse just in the central part of the focus remain excited and contribute to the collected signal. With the special shape and power of the depletion-pulse it is possible to enhance the spatial resolution by one order of magnitude down to only 10 nm and break the diffraction barrier of the excitation light.

Another way to decrease the excitation volume with a resolution comparable to the confocal scheme applies: the simultaneous absorption of multiple photons. This can only occur if the photon density is rather high, meaning the light is compressed in all three spatial dimensions: Two of them by means of a focusing lens with high numerical aperture and the third by means of the ultrashort pulse duration itself. In contrast to conventional confocal microscopy no pinhole in front of the detector has to be inserted to eliminate signal originating from out-of-focus: the intensity for signal generation is only high enough in the tight focus itself. Cumbersome alignment procedures of the pinhole are therefore redundant and the bleaching of markers is restricted to the volume where also signal is detected from.

Another advantage is the much longer wavelength of the excitation light because two-photon-excitation (Fig 1.) works with twice the wavelength compared to one-photon excitation. The required wavelengths lie in the so-called “optical window” (near infrared). This allows looking deeply into organic samples because the red-shifted light is scattered significantly less but not yet strongly absorbed by water like Mid-IR radiation. Furthermore, the cell viability is much higher at longer wavelengths which means that living cells can stand even higher average powers.

Marker-Free Techniques

For many biological questions it is crucial to reduce the disturbance of the studied system to a minimum. Otherwise, the extracted information depends more on the scientific method itself than the actual process under study. The advantage of microscopy is that shining light in an appropriate manner onto the living sample is considered as one of the most non-invasive methods. Nevertheless, fluorescence microscopy employs markers which interfere somehow the life processes: they are attached specifically to organic molecules and disturb their actual functionality – even cause cell death in worst case. Therefore, truly non-invasive microscopical techniques are desired and attract more and more scientists every day. These so-called “marker-free” methods are based on intrinsic properties of the cell compartments. For instance, vibrational fingerprints of molecules lead to highly specific Raman spectra which are utilized as

Figure 1: Two Photon Fluorescence of granule cells stained with dTomato. Laser parameters: 1070 nm, 40 fs, 3.5 mW at the sample. Courtesy of University of Konstanz.

Figure 2: Cross-like DNA-damage induced in the nuclei of HeLa cells via Three Photon Absorption. Laser parameters: 780 nm, 200 fs, 12 mW power at the sample. Visualized with Epi-Fluorescence. Courtesy of University of Konstanz.

The Company

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TOPTICA develops, manufactures and distributes its innovative lasers for scientific and industrial applications. The company with over 80 employees is fully ISO-certified and celebrated its 10th anniversary in 2008. Starting with high-end diode lasers, the portfolio was soon extended to reference drives for data storage, fiber lasers for fs-applications and photonical accessories like wavemeters. TOPTICA is proud to have not only high-tech companies as OEM clients, but also nearly a dozen Nobel Laureates as scientific customers.

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earlier, NIR laser pulses are only absorbed at lower powers are sufficient. As mentioned above, whereas for imaging up to 1000 times higher laser pulse energies – in part about 100 nJ – are necessary whereas for manipulation much higher laser pulse energies are of interest. For many applications not only visualization but also active manipulation of life processes or organic compartments are of interest. Another key requirement is the reliability: in the first place to keep the experimental parameters always the same, secondly to avoid down-time and thirdly to save the time for maintenance.

Demand for Automation

This brief overview over the vast variety of fs-applications in life sciences is not exhaustive and other common points are worth mentioning. Biophotonics moves its focus from delivering a qualitative picture of life processes to give an even more precise quantitative statement about them. Of course feasibility studies still play their role. But for example it is no longer enough to figure out if a drug shows the desired effect but also which pathway is triggered, which success rate is achieved, which timescales are involved and so on. Furthermore, hundreds of identical measurements must be repeated for a reasonable quantitative statement. This takes a lot of time and effort which leads to the demand for automation: Clinical and pharmaceutical laboratories already employ high throughput and/or high content screening with fully automated routines. But also scientists at the cutting-edge research urgently seek for easy-operation and hands-off instruments to get not distracted from their original focus on scientific tasks. In addition, the complexity of biological problems requires an examination with many different approaches. That means – narrowed down to the fs-applications – a single flexible laser source capable of serving most of the techniques would be appreciated very much. Another key requirement is the reliability: in the first place to keep the experimental parameters always the same, secondly to avoid down-time and thirdly to save the time for maintenance.

TOPTICA’s answer to these expectations resulted in a matured product: the FemtoFiber Scientific. First of all, the laser system is a fiber based solution. Only high quality components with extraordinary long lifetimes are built-in. These comply with telcordia specifications and are suitable for...
24/7 operation. The heart of the laser – namely the master oscillator and the amplifier – are all-fiber based which makes alignment redundant and the system extremely robust. On the other hand the modular extensions of the system offer the desired flexibility: For THG and SHG microscopy the wavelengths 1560 nm and 780 nm, for multiphoton microscopy the supercontinuum from 980 to 1400 nm – filling the gap of Ti:Sa lasers – and for confocal microscopy the doubled supercontinuum from 490 to 650 nm are available. The IR pulses for multiphoton techniques can have extremely short pulse durations. Less than 40 fs were measured at the sample position after appropriate pre-compensation for the microscope optics. For CARS microscopy, two perfectly synchronized laser pulses can be independently tuned to cover all important Raman bands. For OCT over 250 nm of bandwidth at 1 µm are offered. For FLIM also one picosecond pulses with narrow linewidths of only 2 nm can be tuned over the whole visible spectral region. Mainly newcomers to fs-applications feel insecure whether the power of some tens or hundreds of milliwatts is enough for microscopy. But it has already often been proven by scientists that just a few milliwatts delivered at the living sample are sufficient and are even better for its viability. For practical reasons no water cooling, no nitrogen purging, no high power consumption and only a small space on the optical table are necessary. Finally easy operation, hands-off functionality and best price-performance ratio complete the picture.