Multimodal imaging paves the way forward in life sciences

Thomas Hellerer, Toptica Photonics AG, Gräfelfing/Munich, Germany

New microscopy methods find their application in life sciences, such as in metabolic research. The analysis of life processes requires the organisms to be fully vital and intact, thus the imaging should affect the samples as little as possible. Therefore, non-invasive methods are the first choice: among these multimodal imaging gathers multiple signals generated by either SHG, THG, CRS or auto-fluorescence – each of them delivering complementary information.

Today adipositas is one of the major lifestyle diseases. For this reason, many researchers focus on the metabolism and how to influence it positively. In contrast, to standard methods in cell biology, it is not sufficient to examine only single cells or cell structures. The complex interplay of regulatory mechanisms like the discharge of insulin requires a fully vital and intact organism. For the sake of simplicity only very simple and well understood organisms are investigated, which nevertheless enable conclusions about humans to be drawn.

Living organisms as sample objects

The worm Caenorhabditis elegans is a well-established organism in metabolic research. The 1-mm long worm lives on plants and is genetically fully analyzed [1]. Already, valuable insights could be gained on basis of different mutants. No longer than seven years ago the worms were still examined with a centrifuge in order to determine their fat content. Then, a technique was found to label their food and to observe the worm's food uptake while it was still alive [2]. This new step was a breakthrough for observing the intact organism and even enabled the location of the fat distribution within the worm. Unfortunately, later it turned out that this method does not tell the entire truth: the markers don't label the entire fat of the worm but only a certain part of it [3]. This shortcoming is exemplary for the major problems in fluorescence microscopy, which relies on markers to label the structure of interest. Another problem arises from the fact that the labelling itself changes the functionality of the labelled cellular compartments. This has far-reaching consequences for the analysis of life processes. This is why scientists seek new methods to eliminate the disturbing influences – despite the justified big success of the fluorescence technique. The key are non-invasive methods, which avoid special preparation of the samples. Instead, 'natural' signals deliver the desired information with high specificity.

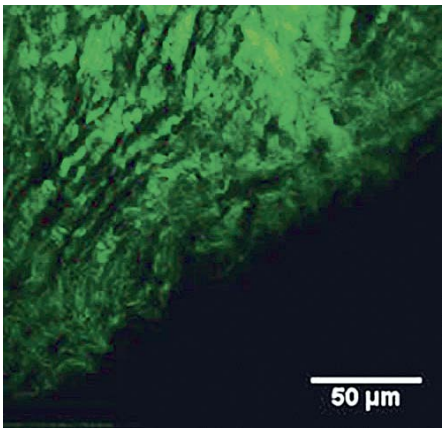


Figure 1: SHG-Image of collagen fibres in a sample of a human artery
(courtesy: Group of Molecular Microscopy, Chalmers University)

Non-invasive methods and non-linear effects

Several non-invasive methods were developed during the last decade, many of which were also applied successfully to biological problems [4]. Among others, there are second (SHG) (**figure 1**) and third (THG) harmonic generation microscopy and coherent Raman scattering (CRS) microscopy. All these techniques are based on non-linear optical processes. For these techniques to be employed, very high powered lasers are required that would kill the cells if operated in continuous wave (cw) mode. Therefore, ultrafast lasers are used, which emit in a tiny fraction of a second (10^{-13} s) a laser pulse with high peak powers of several tens of kilowatts. On the other hand, long pauses between the ultrashort laser pulses reduce the overall average power to moderate levels, which the cells can tolerate.

Non-linear optics includes many interesting effects, several of which can be easily explained by the photon picture. Imagine the emission of the laser as a stream of photons with a density proportional to the light intensity. If the density is high enough, the probability is also high that two photons "meet occasionally" in the stream and interact via the optical nonlinearity of the sample. In this way, two photons can annihilate and create a new photon with the energy doubled and the wavelength cut in half. This process is termed as second harmonic generation (SHG). Only samples which possess a second order nonlinearity, generate a signal at half the wavelength of the laser. This feature can be employed as contrast mechanism for imaging making the markers redundant. In living cells mostly stiff structures like protein filaments (microtubules) and collagen fibres are sources of the SHG-signal. Third harmonic generation (THG) is analogous to SHG but with three photons transformed into a new one at one-third of the laser wavelength. The main source of the THG-signal in living organisms are lipid droplets (= fat). Both optical processes – SHG and THG – take place simultaneously. The very same laser pulses cause the different cellular compartments under the microscope to shine with either one-half or one-third of the laser wavelength. The simultaneous use of both signals, which carry different structural information, formed the term 'multimodal imaging'. The microscope operates with the same light source in two modes, the SHG-imaging and the THG-imaging at the same time. The huge potential of this method can be extended even more by adding additional light sources and dectectors.

For example, an extension of the light source can add CRS (**figure 2**) as fur-

Originally published in German in BioPhotonik 2/2010

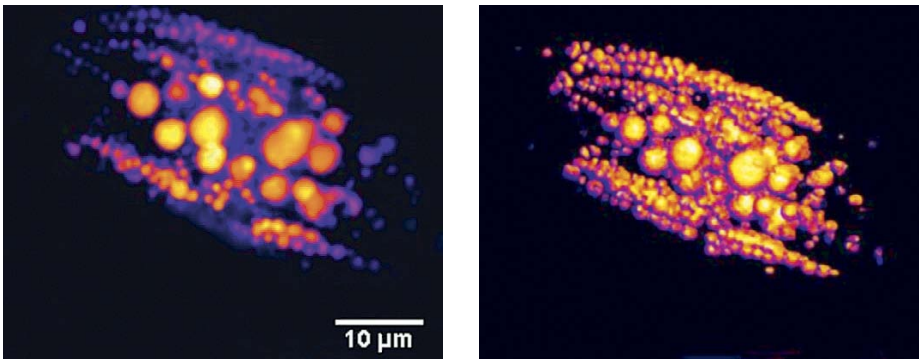


Figure 2: CARS (Coherent Antistokes Raman Scattering) image of lipid droplets from the central part of a living worm (C. elegans). Left: a single optical section through the worm. Right: a 3D-rendered image, calculated from many optical sections *(courtesy: Group of Molecular Microscopy, Chalmers University)*

ther imaging mode: two synchronized and pulsed lasers with different wavelengths enable three photons to interact with each other generating a fourth one which then is detected. This mode features the freedom to choose any cellular compartment for contrast generation by tuning the wavelength difference of the lasers accordingly. The underlying physical mechanism is based on the vibrations of the molecules which build up the cellular compartments. The Raman effect describes the interaction between these molecular vibrations and the laser light. The involved (Eigen-) frequencies are specific for each type of molecule, and can be excited coherently by the difference frequency of both lasers. By tuning the lasers to the specific molecular frequency, these molecules enhance the generation of the coherent Raman signal (coherent Raman scattering, CRS). For practical reasons the CRS method favours the imaging of lipid droplets, mitochondria, membranes and proteins because of their high Raman activity.

The three optical processes presented above are joined by the auto-fluorescence of certain cellular compartments like hemoglobin, melanin, elastin or the coenzyme NADH, which plays an important role in the cellular energy balance. Despite the fact that they are necessarily dyes like the markers used in labelling, they occur naturally in cells and therefore do not disturb their proper function. If they are not excited with visible light, as it is normally the case, but excited with pulsed infrared lasers, two photons instead of one photon carry enough energy for their excitation (analogous to SHG). The advantage compared to visible excitation is that the high intensity that is necessary for two-photon absorption is confined to the tight focus of the microscope objective. The fluorescence signal of the dye thus originates only from a single spot within the cell. This focal spot is scanned with two mirrors layer by layer through the sample while the signal is detected pixel-wise. The subsequent 3D-analysis reconstructs the three dimensional structure of the imaged organism. Even arbitrary optical sections can be displayed. This 3D and optical sectioning capability is intrinsic to all non-linear microscopy methods and otherwise requires the technical effort realized in confocal microscopy. **Table 1** gives an overview of the four presented non-invasive methods – SHG, THG, CRS and auto-fluorescence. They are the facets of multimodal imaging and are all based on non-linear optics.

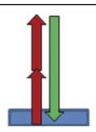
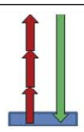
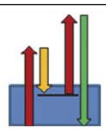
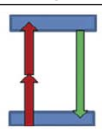
Method	SHG	THG	CRS	2-photon autofluorescence
No. of photons	2+1	3+1	3+1	2+1
Cellular compartments	microtubules, collagen fibres	lipid droplets	lipid droplets membranes, proteins, water	elastin, NADH, melanin, hemoglobin
Energy level scheme				

Table 1: Overview of non-invasive and optically non-linear microscopy methods

Outlook

The promising approach of multimodal imaging is becoming established in life sciences. This is made possible on one hand by modern microscope objectives that are now optimized for infrared light. On the other hand ultrafast fibre lasers have been readily available for a few years, and fibre lasers do not require technically skilled scientists for operation and can be run by non-technical personnel in clinics or bio-facilities. These ultrafast lasers are based on rare-earth doped fibres as gain media and saturable absorbers for stable pulsed operation. These compact laser systems require neither water cooling nor heavy-current lines and are therefore easily integrated into life science laboratories (**figure 3**).

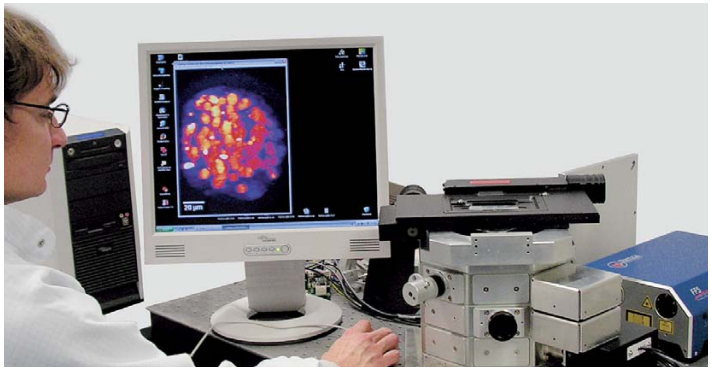


Figure 3: Fibre laser attached to a multimodal microscope *(courtesy: Till Photonics GmbH)*

Literature:

[1] Science 1998; 282(5396):2012-8
[2] Nature 2003; 421:268–272
[3] PNAS 2007; 104:14658-63
[4] Opt.Exp.2009; 17:1282-90

Author contact:

Dr. Thomas Hellerer
Senior Sales Manager
Ultrafast
Toptica Photonics AG
Lochhamer Schlag 19
82166 Gräfelfing/Munich
Germany
Tel. +49/89/85837-184
Fax +49/89/85837-200
eMail: thomas.hellerer@toptica.com
www.toptica.com