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Research Newsletter – European Edition

# reSOLUTION

## **From Molecules to Tissues**

Optical Tools for Cancer Research

## **Closing the Spectral Gap**

The First Supercontinuum Confocal that Adapts to the Sample

## **Obese and Slim Yeast Cells**

Microscopic Insights into Cellular Lipid Metabolism



Dear Readers,

Only twenty years ago, it was generally thought that the optical potential of light microscopy was exhausted. Fluorescence microscopy had already brought significant advances in cell biology, particularly for genetics and cancer research, but there were still limits to optical resolution. Abbe's law of diffraction seemed to be chiselled in stone.

Confocal laser scanning microscopy paved the way for new groundbreaking technologies such as 4Pi and especially STED microscopy. Attaining unprecedented nano-scale resolutions, they finally enable the visualisation of molecular structures that are crucial for understanding the processes of life. Today, light microscopy is more important in life science than ever before. And what's more, developments are still in mid flow – the future is sure to bring more exciting solutions in terms of speed, sensitivity and resolution.

In this issue we present another technology that opens up new horizons for research scientists and underlines once more the leading innovative role played by Leica Microsystems: the first supercontinuum confocal with white light laser and AOBs for full emission and excitation flexibility. The possibilities of combining light microscopy with other microscopic techniques are equally exciting. An example featured here unites atomic force microscopy and light microscopy in one instrument.

As we want to keep on writing about the topics you are interested in, we always welcome your opinion. Turn to page 19 for further details.

Have fun reading!

Anja Schué  
Corporate Communications

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Cover picture: Vascular anatomy of a zebrafish embryo as revealed by GFP expression driven by the Fli-1 promoter. Courtesy of Dr. Brant Weinstein, National Institutes of Health, Bethesda, MD.

## Optical Tools for Cancer Research

# From Molecules to Tissues

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### The postgenomic era of fluorescence microscopy: molecules, cells and tissues

Sequencing of the human genome stimulated a radical change in the approach to biomedical research. The comprehension of the mechanisms regulating life gained a scale-up in throughput to speed up the retrieval of data for a global vision of a system of incomparable complexity. The birth of the genomic sciences sparked the proliferation of “sister” approaches: starting from single genes it is now necessary to identify and characterise their products (proteomics) to reveal their role in the targeted systems, namely the living cell (cytomics). Purely qualitative observation typical of the pre-genomic era turned into a more quantitative approach to provide the input data for more and more complex analysis aimed at simulating the network of biochemical reactions ruling life.

Oncological research has been deeply involved in this change process, recognising the risks of a sterile approach based solely on *in vitro* models. The need for therapies targeting specific molecules realises a *trait d'union* starting from analysis of single molecule biochemical structures and extending to their role in the cellular environment both at intra- and intercellular level. The analysed scenario subsequently evolves from clonal tumour populations to the intricate network of interactions established between the tumour and the host first examined in tissue biopsies and then in living animal models.

Modern optical microscopy has always represented an indispensable tool for biomedical research and has developed to adapt to the continuously chang-

ing requests dictated by the great heterogeneity of samples targeted by experimental approaches. Besides a somehow obvious need to progressively increase resolution, optical microscopy must now extend its non invasive analytical ability from single cells to entire organism minimising the disturbance to life during the observation step.

The revolution of the multiphoton excitation microscope represented a big step toward the observation of complex living systems [1]. Infrared high frequency sources provided an incomparable penetration depth. The ability to easily induce fluorescence from UV excitable molecules has been successfully employed to monitor concentration and functionality of metabolic and structural markers *in vivo*, such as NADH and collagen respectively. The developed assays supported the pioneer involvement of two-photon microscopes in clinical trials to realise a first approach to “optical biopsies” in the diagnosis of skin neoplasia [2].

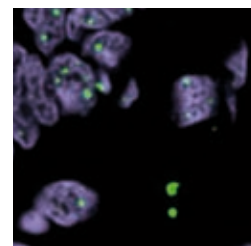
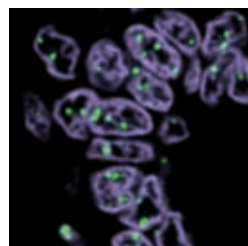
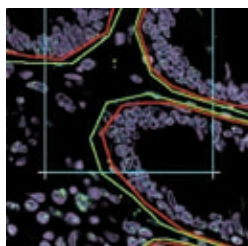
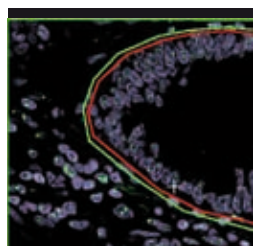
One of the most relevant and successful transformations of the modern microscope is the ability to parallel an instrumental modification with the development of new assays for the determination of functional parameters. Besides the emerging field of nanoscopies, characterised by the birth of 4Pi and STED microscopes allowing the reduction of the gap in spatial resolution between optical and electron microscopy, our insights into the properties of single molecular species gained relevant advantages from the birth of new functional microscopy assays and techniques. This process culminated in the rediscovery of the “F Techniques”, namely Fluorescence Recovery After Photobleaching (FRAP), Fluorescence

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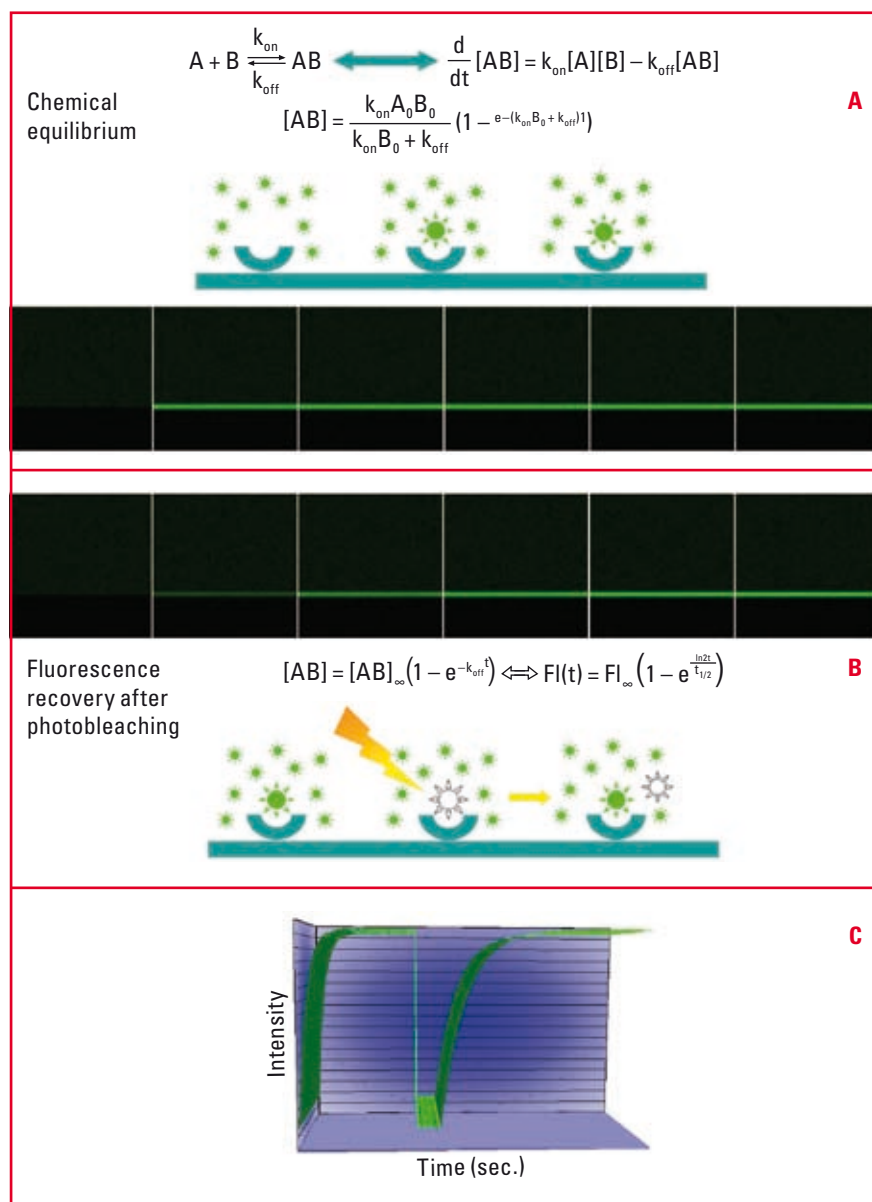


Fig. 1: *In vitro* fluorescence recovery after photobleaching for characterisation of chemical reaction kinetics (courtesy of A. Musacchio, M. Vink).

A: Temporal evolution of the concentrations of the components in a chemical binary system is modelled by differential equations with straightforward analytical solution under proper experimental conditions. One of the two species is immobilised on a surface (coverslip, agarose beads) while the other is covalently tagged with a fluorescent molecule. Formation of the complex is accompanied by accumulation of a fluorescence signal on the coverslip. The temporal behaviour of the fluorescence intensity is ruled by association and dissociation rates depending on the concentration of ligand in solution.

B: Recovery of fluorescence after photobleaching of the surface is completely ruled by dissociation rates under the assumptions of no-rebinding and fast diffusion when compared to chemical kinetics. Half-recovery time coincides with the half-life of the studied molecular complex.

C: The graph reports the evolution of the intensity of the fluorescence signal. The first phase is characterised by reaching of chemical equilibrium. After that, complexes on the surface were bleached and recovery observed. The different temporal behaviour of the two phases can be consequently appreciated.

Resonance Energy Transfer (FRET) and Fluorescence Correlation Spectroscopy (FCS) for the study of molecular dynamics and interactions.

In the following, we will focus our attention on specific applications, providing some examples of how a “standard” confocal microscope can vehicle oncological research across growing levels of complexity. Starting from the properties of single isolated molecules, we will then move to their role in the living-cell biochemical network, scaling up from statically homogeneous samples (*in vitro* cell cultured populations) to the intrinsic heterogeneity of the histological analysis.

### Molecules: functional nanoscopies. Characterisation of chemical reaction kinetics

The study of complex biochemical networks starts from simplified experimental models as *in vitro* binary systems constituted by purified biomolecules. Till now, measurements of chemical reaction kinetics parameters were obtained by isothermal calorimetry and/or surface plasmon resonance based instrumentation, with high acquisition and maintenance costs.

Confocal microscopy and, in particular, Fluorescence Recovery After Photobleaching (FRAP) protocols can provide an efficient alternative as recently published in a paper on the characterisation of the molecular mechanisms at the base of the “Spindle Assembly Checkpoint” [3]. In FRAP protocols photobleaching is employed to inactivate fluorescence molecules inside target regions. Molecular mobility leads to recovery of the fluorescent signal by replacement of the bleached fluorescence molecules. The speed of signal recovery represents an index of molecular mobility determined by the diffusion coefficients in the environment where molecules reside (the different cellular compartments) and by the interactions taking place with other molecular species. When a single molecular species is immobilised on a surface and exposed to a second interacting molecule fluorescently tagged in solution, an increase of emitted signal will be detected till reaching of the chemical equilibrium (Fig. 1).

The temporal evolution of the intensity will be ruled by the association and dissociation rates proportionally to the concentration of the reactants in the system. However, the recovery of fluorescence in a photobleached region of the above mentioned surface will no longer depend on such parameters. Under proper assumptions, i.e. that the diffusion process took place on a time scale much faster than the



chemical reaction, the limiting step in the replacement of a bleached molecule is the disassembly of an existing complex. Half-recovery time consequently coincides with the half-life of the studied molecular complex. Comparison of the graph of the fluorescence temporal evolution revealed the differences of kinetics of the two processes: reaching of chemical equilibrium happened on a faster time scale, while photobleaching did not disturb the gained chemical equilibrium and is consequently ruled out by the half life of the existing complex only.

### Cellular networks: highlighting molecular fluxes

The living cell provides a perfect target for validation of FRAP protocols. Coordination of the mechanisms ruling life is accomplished through a perfect degree of spatial and temporal compartmentalisation. Besides FRAP techniques, our ability to follow molecular motion gained significant advantages with the cloning of the photoactivatable/photoconvertible variants of fluorescent proteins. The possibility to selectively switch on fluorescence, creating a highly localised increase in signal-to-noise ratio, enormously facilitated molecular and cellular tracking in living cells and organisms. A strong limitation in photobleaching/photoactivation protocols based on confocal microscopy derives from the inability to control the depth of the targeted volume: a focused Gaussian laser beam will essentially deliver high energy density sufficient to photoactivate/photo-bleach molecules inside the illumination cone of the objective spanning over several microns.

The use of optical setups with intrinsically limited excitation opened the way to full control of the photoconversion process in three dimensions [4, 5]. Total Internal Reflection Fluorescence Microscopy (TIRF) is able to photoactivate molecules on a cell region spanning some hundreds of nanometres over the basal membrane employing the energy delivered by an evanescent electromagnetic field created at the interface between two media with different optical properties, namely the glass coverslip and the cell membrane. Two-photon microscopy is able to move inside the cell, targeting volumes down to a diffraction limited extent. The possibility to confine the activated volume in three dimensions provided new tools for some challenging questions in living cell analysis. Targeted photoactivation of endocytic vesicles without involvement of the upper and lower membrane, only feasible with two-photon induced photoconversion, can be successfully employed to demonstrate the existence of recycling molecular fluxes directed from the cytoplasm to the cell membrane.

### Tissues: confocal histopathology in three dimensions

Classical histological analysis has been based for years on the optical microscope, entering only marginally the field of fluorescence microscopy. The evolution of modern confocal microscopy and in particular the birth of spectral detection systems, the high degree of automation and the non-linear excitation revolution recently stimulated and strengthened interactions between Laser Scanning Confocal Microscopy and histological analysis [6]. Histopathological samples represent a first step towards a rescaling in the complexity of the analysed material required by the postgenomic revolution. This complexity turns the richness of material into a hard environment for light propagation. Light scat-

Fig. 2: Spatial confinement of one- and two-photon mediated photoactivation applied to molecular fluxes towards plasma membrane.

A: Focused Gaussian laser beams deliver excitation energy over a volume spanning throughout the entire cell thickness. Targeting of intracellular compartments without involvement of upper and lower membrane portions is consequently unfeasible, as demonstrated by the temporal evolution of the signal reported in the bottom of the panel.

B: Two-photon excitation induces localised photoactivation able to separately target inner compartments such as internalised enlarged endosomes without extending to the plasma membrane. The time evolution of the signal intensity underlines the transport process recycling part of the molecules as evidenced by the temporal shift observed in activation of the membrane areas with respect to the targeted vesicle.

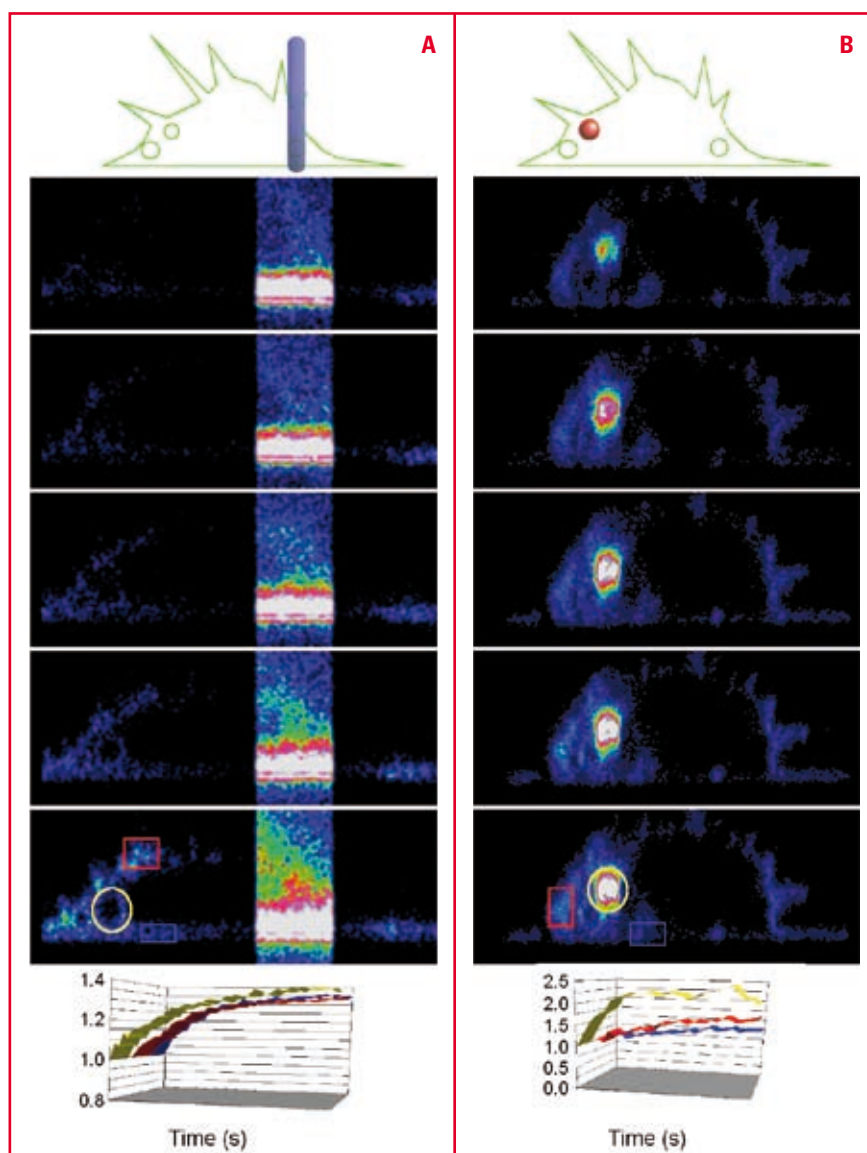
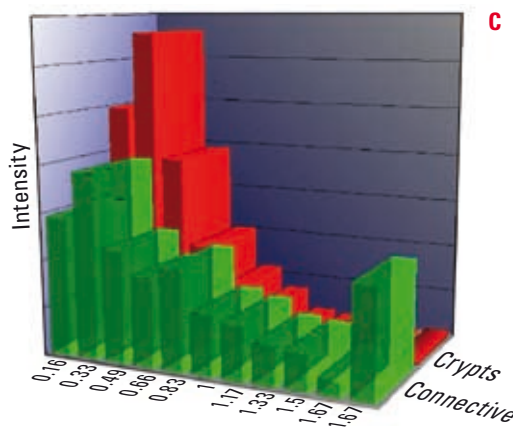
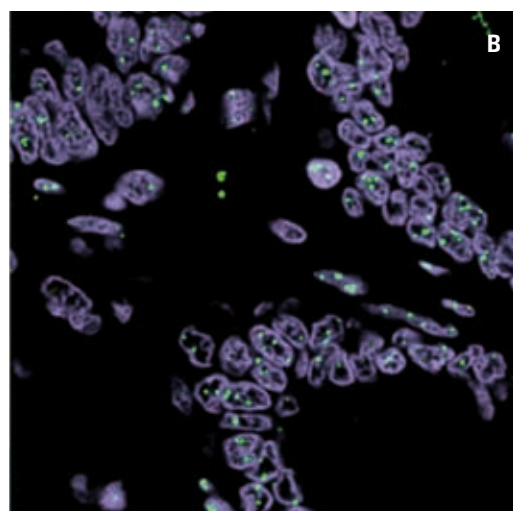
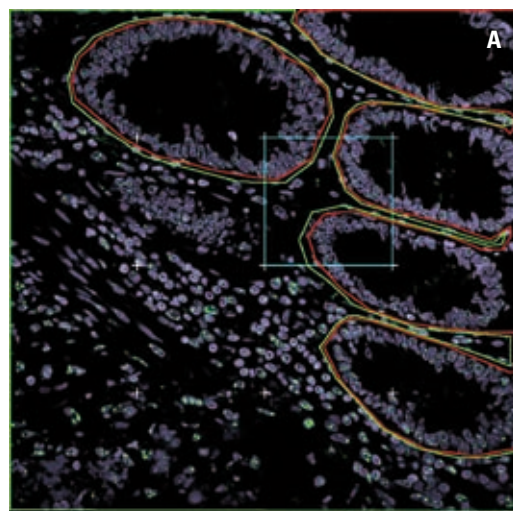


Fig. 3: High resolution confocal histology.

Mosaic acquisition (A) of an immuno-stained human intestine tissue section. Sequential recording of single images with a pixel size (80 nm) compatible with Nyquist rate (B) allowed analysis of intracellular details in diffraction limited conditions and subsequent correlation to the histological features of the sample over the reconstructed field of view (around 400  $\mu\text{m}$ ). Measurement of the mean size of the nuclear bodies where the PML protein is located revealed histological compartmentalisation (C). Crypts (red line and regions in A) showed a quite homogeneous distribution peaked around 250 nm lower than the results measured in the surrounding areas (green line and regions in panel A), where the distribution spread over a wider range of values.



tering can compromise the signal-to-noise ratio making the real resolution extremely poor.

This problem can be solved by coupling the optical pinhole of the confocal microscope with the “digital pinhole” created by the computational techniques of image restoration commonly known as “deconvolution”. Another type of deconvolution can be ex-

tremely useful when analysing such complex samples, namely spectral deconvolution. Measurement of the photophysical features of the analysed material can help both in understanding tissue composition and in setting up the best fluorophore combination for maximisation of the analysable parameters in a high content analysis.

The next step consists in obtaining a diffraction limited observation for determination of intracellular parameters over extremely wide fields of view allowing for the recognition of the distinctive tracts of the tissue organisation. The realisation of such a challenging task requires a mosaic approach that can be exploited either by use of automated stages or by intelligent use of the scanning mirrors of the confocal microscope. Such an approach has been adopted to measure the size distribution of ProMyelocytic Leukemia protein nuclear bodies in different histological compartments in a biopsy from a normal human intestine (Fig. 3). The field of view ( $360 \times 360 \mu\text{m}^2$ ) has been obtained by merging 16 different panels collected with a sampling pixel of about 80 nm. Such an analysis suggested a correlation between intracellular properties and histological compartmentalisation. *In vitro* studies attributed to PML an anti-proliferative effect. Mean size and intensity of PML nuclear bodies in the intestine crypts is lower than the values calculated in the surrounding connective tissue, suggesting a correlation between protein content and proliferation, the crypts being sites of high replicative activity.

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# The First Supercontinuum Confocal that Adapts to the Sample

## Closing the Spectral Gap

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Until now, biological and medical research fluorescence imaging in multi-user facilities or institutes has been limited by the type or number of dyes that could be excited. The Leica TCS SP5 X supercontinuum confocal unites the broadband capabilities of the Leica TCS SP5 AOBs® and the freedom and flexibility to select any excitation line within the continuous range of 470 to 670 nm.

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- 2 LAMBS-MicroScoBio Research Center, Department of Physics, University of Genoa, 16146 Genoa, Italy
- 3 Leica Microsystems

Fig. 1: Paramecium stained with eight fluorescent dyes. Excitation and emission lambda scans were obtained to determine optimal imaging conditions for each dye. Courtesy of P. Ramoino and P. Bianchini, LAMBS-MicroScoBio, Dept. of Physics, University of Genoa, Italy.

With the new Leica TCS SP5 X and its white light laser source the user has complete freedom to choose the detection area in up to five spectral confocal PMT channels. The active acousto-optical beam splitter AOBs® allows the selection of up to eight simultaneous lines from anywhere in the white light spectrum (Fig. 1). Optimal adjustment of the excitation line to the sample – in 1 nm increments – reduces cross-excitation and minimizes sample damage (Fig. 2). The system precisely adapts to any existing or future fluorescent dye. With 200 nm of freely tunable excitation lines, it can be tuned to provide optimal excitation of any available dye. This flexibility is critical for core facilities that service hundreds of users with various samples and various dyes.

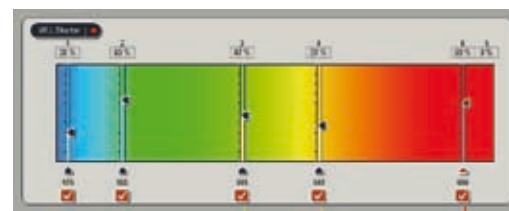
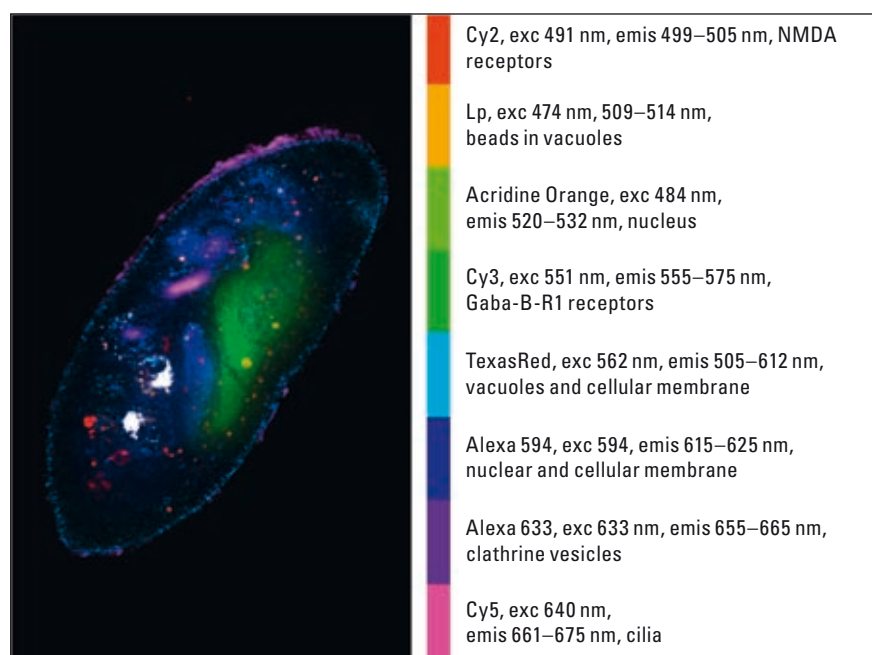


Fig. 2: Intuitive interface allows full freedom and flexibility for scanning:

- Excitation lines can easily be selected to any wavelength with 1 nm accuracy
- Tuning via the LCD control panel allows selection of excitation light while scanning
- Easy intuitive interface makes the Leica TCS SP5 X perfect for basic and advanced users
- Interface allows easy access to all scanning parameters for both supercontinuum and fixed laser lines

### Tune in to the excitation optimum

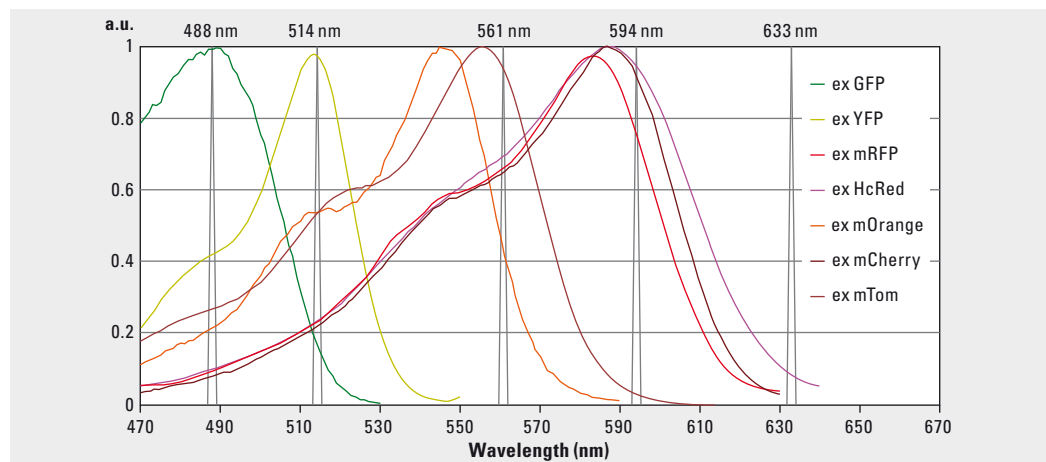
If comparing excitation spectra and available emission lines of conventional lasers, the gap is obvious: most dyes cannot be excited at the position of their maximum cross section. A common dye like Alexa 488, although it is designated as “488 nm” dye, has an excitation maximum at 500 nm, whereas the absorption at 488 nm is only 75 %. The Leica TCS SP5 X allows users to steplessly tune the excitation, no matter what the name of the fluorochrome is suggesting. So the fluorochrome can be excited at its maximum cross section, for Alexa 546 this would be 561 nm (Fig. 3).

Nevertheless, this is not necessarily the best position of excitation. In order to prevent excitation light from entering the detector, a certain “security distance” has to be kept between excitation and the blue edge of the emission band that is collected. If the Stokes shift is rather low, then the residual window for emission collection might cut off a significant part of the available photons, which is not desirable. So it sometimes does indeed make sense to excite the dye somewhat off the peak in the blue range (Alexa 488 case) and compensate for the lower absorption by increasing the laser intensity.

The combined operation of tunable excitation and tunable emission can help to find the best set-



Fig. 3: Excitation spectra of various fluorescent proteins and excitation lines of commonly used conventional lasers (ROYB-type). Obviously, most of the shown FPs can be excited optimally with the laser combination indicated. Courtesy: K. Jalink, Netherlands Cancer Institute, Amsterdam.



ting for excitation and emission: a software tool is available that acquires images at incrementing excitation wavelength (excitation scan) and also adjusts automatically the blue cut off of the emission band, for example always 10 nm off the excitation to prevent reflected light from entering the detector.

### Reduce crosstalk easily

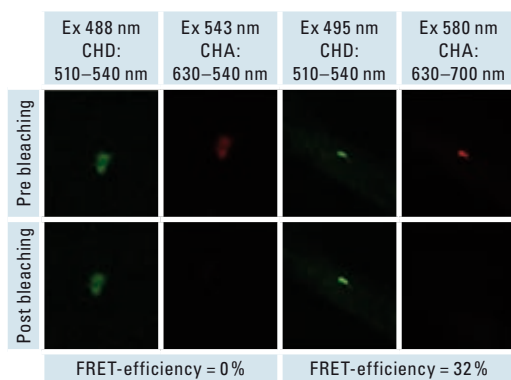
A common challenge with multiparameter fluorescence is that excitation even by a narrow line will excite not only the targeted dye, but also other dyes in the sample. In most cases this is not wanted as the separation of various channels suffers from cross excitation making some applications, like FRET experiments, difficult to work with. Here again, the tunable wavelength of the white light laser source is an easy cure for cross excitation. The longer wavelength excitation can be moved out of the absorption of the blue dye. Here, an interactive optimisation for reduced cross excitation and efficient emission collection is easily done by dialing the excitation and adapting the emission band – online by a few scans only.

If separation is still not optimised, the system also offers sequential scan: that is recording a line with the first excitation and subsequently the same line with the next excitation for the next dye – and so on. Furthermore, the SP detector as a tunable device can be specified to collect sufficiently narrow bands for minimal crosstalk on the emission side. If the above procedures still do not provide optimal separation, linear unmixing for dye separation – emission or excitation – is also implemented.

### Optimised FRET

As an example, a FRET AB (FRET by Acceptor Bleaching) experiment with the FRET pair Alexa 488 and Alexa 568 is described. This is only one out of a long list of possible FRET pairs, but commonly used. The bleaching of the acceptor is performed by the 543 nm HeNe laser line, if there is no better choice available. According to officially published data, the absorption of the donor is sufficiently low (<2 %), to not interfere with the acceptor bleaching. The experiment was done as mentioned, and as a result, no increase of the donor was detectable, so one would conclude that the proteins are not “colocalised”, and at least 10 nm apart. When measuring the excitation spectrum of the donor *in situ* by a supercontinuum confocal, it turned out that the absorption at 543 nm is indeed much higher, about 11 %. When the same experiment was done with laser light tuned to 580 nm for the acceptor, the donor emission increased significantly by approx. one third – corresponding to 33 % FRET efficiency. 580 nm is sufficiently away from donor excitation, so the donor is not bleached during acceptor bleaching and will emit more fluorescent light after the FRET partner is removed. The lack of increase in the previous experiment was due to bleaching of donor by the laser light applied for acceptor bleaching (Fig. 4).

Fig 4: FRET AB measurements. FRET efficiency is measured by increase of donor emission after photobleaching of acceptor. In the left example with conventional lasers, donor emission is not increasing (green images in the left row). The right side shows a strong increase in donor fluorescence using a WLL tuned excitation for acceptor. Courtesy: V. Caorsi, A. Diaspro, Genoa.





## Excitation properties *in situ*

The white light laser source in combination with an AOTF as fast programmable line selector provides a very straight forward of generating excitation spectra from dyes *in situ*. The software allows for automatic incrementing of the excitation wavelength while the scanner takes images at each  $\lambda$ -position. The standard operation will keep a preset emission band; the result is a direct measure for excitation dependence of the dye as a function of wavelength. A second operation mode will move the blue edge of the emission band synchronously with the excitation in order to always record the maximally available fluorescence. This is especially valuable for screening new or unknown dyes to find out the best excitation emission settings. Evaluation of excitation spectra is easily possible by just drawing regions of interest into the recorded images. The software will provide graphs of fluorescence for all regions. As the measurement is done in the sample directly, the results are much more reliable as compared to published data. Series of spectra with varying conditions of the solvent can be measured to find out about spectral changes, e.g. at various pH levels.

## Fluorescent proteins excitation spectra *in situ*

A set of new fluorescent proteins was examined by excitation scans. The data may be used to optimise excitation and reduce crosstalk when recording images with those FPs. Also, this information is needed when planning FRET pairs. And in general, one can also study changes in fluorescence parameters in living cells and under controlled conditions (Fig. 5).

## $\lambda^2$ -maps on cyanobacteria in Roman catacombs

As an example, the fluorescence spaces of vital cyanobacteria were measured. The specimen was a cyanobacterial film that was collected in a Christian catacomb of the Via Appia Antica in Rome. The microbial community is formed by different filamentous and chroococcal cyanobacterial species, together with bacteria. The spectral differences in the cyanobacterial cytoplasm are due to the intrinsic content in photosynthetic pigments of the three main types chlorophylls, phycobiliproteins and carotenoids. These pigments show species-specific variations in excitation and emission spectra, which help to identify types and physiology of the cyanobacteria. The cyanobacterial mats are identified as destroyers of Roman hypogeal monuments.

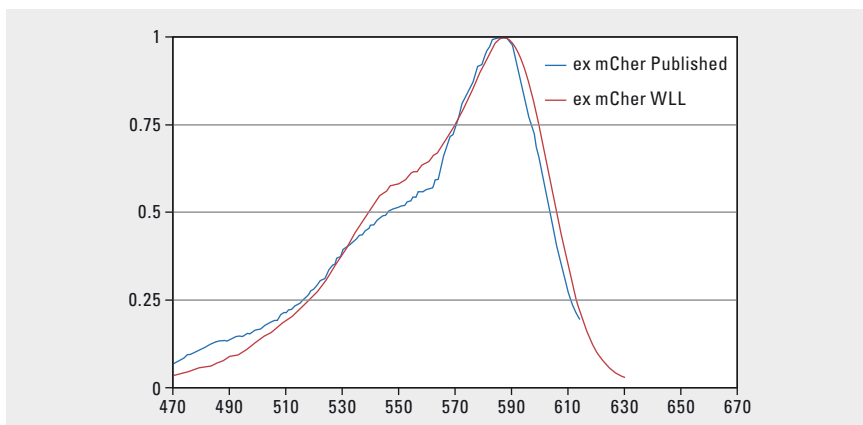


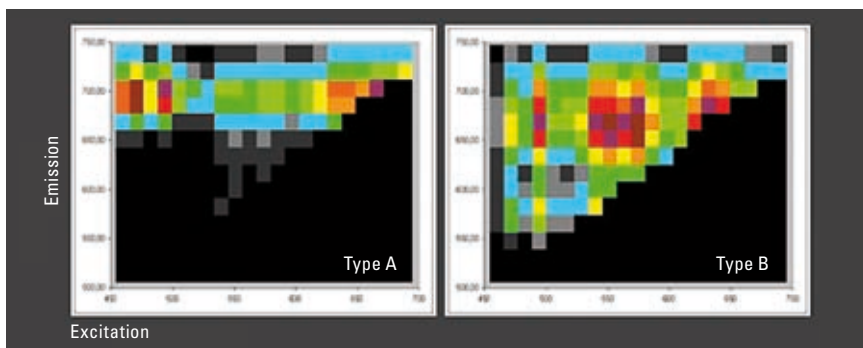
Fig. 5: Comparison of published data for excitation of mCherry and excitation scan with WLL. Courtesy: K. Jalink, Netherlands Cancer Institute, Amsterdam.

Spectral correlation data were taken from a single optical layer by tuning the supercontinuum laser between 470 nm and 670 nm and recording emission spectra between 515 nm and 720 nm. Subsequent analyses of single individuals in regions of interest through the whole four-dimensional sequence reveal species specific intensity patterns. The intensities were normalised and color-coded to visualise the patterns: Very obviously, the two selected types differ in both excitation and emission properties. Upon blue excitation, Type A emits only at longer wavelengths, a very significant emission between 600 nm and 700 nm in Type B is rather absent in Type A. Many more details can be found and evaluated from data at higher spectral resolution (Fig. 6).

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Fig. 6: Excitation – Emission maps of fluorescence from biofilms. Two different species of cyanobacteria are presented and show variant patterns of fluorescence.



## New Standards in Fluorescence Stereomicroscopy

# FusionOptics<sup>TM</sup> meets TripleBeam<sup>TM</sup>

Kristina Mayer and Daniel Göggel, Leica Microsystems

The Leica M205 FA and M165 FC stereomicroscopes are the latest addition to the innovative M series for demanding fluorescence applications in developmental, molecular and cell biology. The microscopes unite top performance zoom optics, resolution and contrast to produce brilliant fluorescence images with outstanding richness of detail.

Combining the revolutionary FusionOptics<sup>TM</sup> with the successful TripleBeam<sup>TM</sup> principle, the fully automated Leica M205 FA creates fluorescence images of exceptional quality. The left optical beam path of the microscope produces high depth of field while the right beam path with its highest possible aperture depicts a high-resolution image. FusionOptics<sup>TM</sup> (patent pending) takes advantage of a neurological phenomenon: through the human brain's capacity to selectively process information gathered with the single eyes, two apparently contradicting worlds are brought together: a three-dimensional, detail-rich image and concomitant depth of field that has been unfeasible with the classical stereo-optics approach. With its fully apochromatic optics, the largest zoom range on the market (20.5:1) and the top resolution performance of up to 1050 lp/mm, the Leica M205 FA is able to show the observer details that used to be invisible.

Fig. 1: The new high-performance fluorescence stereomicroscopes Leica M205 FA and M165 FC

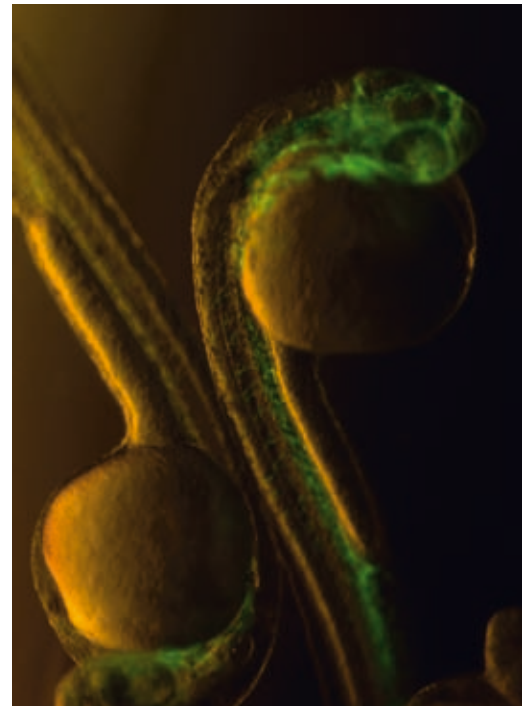


Fig. 2: Vascular anatomy of a Zebrafish embryo as revealed by GFP expression driven by the Fli-1 promoter. Courtesy: Brant Weinstein, National Institutes of Health, Bethesda, MD.

### Fluorescence at its best

The TripleBeam<sup>TM</sup> principle, with its patented third beam path reserved exclusively for fluorescence illumination, delivers evenly illuminated, reflex-free fields of view at all zoom settings. Besides this, the FluoCombi III<sup>TM</sup> objective revolver features the unique capability to exploit all the advantages of both stereo and high-resolution micro-objectives on one instrument with a simple switch. Switching to the parcentric and parfocal micro-objective allows for parallax-free imaging to the finest detail.

Time-intensive studies of living organisms and documentation of complex image series and multi-fluorescence images are made possible and in-

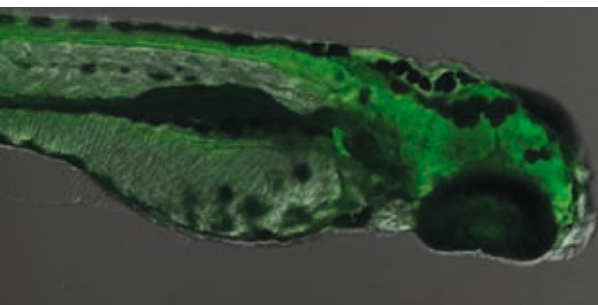


Fig. 3: Zebrafish embryo expressing GFP under the control of the beta-actin promoter. Courtesy: Prof. Dr. Stephan C. F. Neuhauss, Professor for Neurosciences ETH Zurich and Institute for Brain Research at the University of Zurich.

stantly reproducible by motorising focus, zoom, filter changer, iris diaphragm, fluorescence intensity manager and microscope stage. The Leica M205 FA is fully integrated in the modular software solutions Leica AF6000 E to AF6000.

### Manual stereomicroscopy of the highest order

With the Leica M165 FC, the classical stereo-optics approach has been exploited to the utmost optical limits. The fully apochromatic corrected 16.5:1 zoom – combined with TripleBeam™ and FluoCombi III™ – guarantees high-contrast fluorescence images down to the finest structures of the specimen. Encoded zoom, iris diaphragm and objective revolver allow configuration parameters and optical data to be reproducibly read out at the computer.

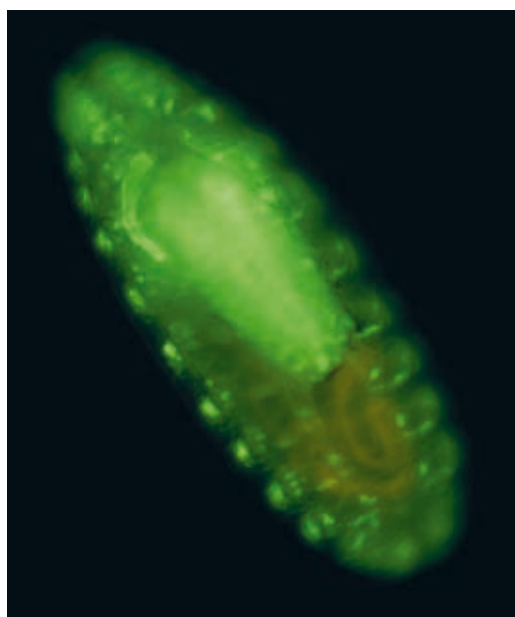


Fig. 4: Peripheral and central nerve (ventral cord) system of a *Drosophila* embryo, salivary gland.

## FusionOptics™ – Nature's ingenuity showed the way

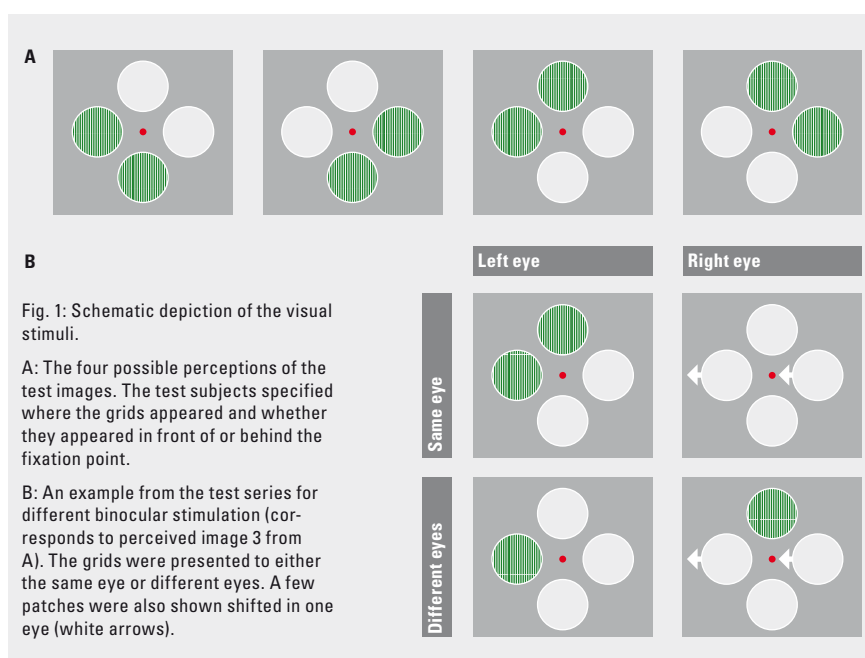
Stereomicroscopes used to be designed according to physical principles based primarily on Ernst Abbe's work. The limit of what was optically possible was determined by the correlation between resolution, convergence angle and working distance. The higher the microscope resolution, the greater the convergence angle between the left and right beam paths and the lower the available working distance. However, increasing the distance between the optical axes causes the three-dimensional image seen by the observer to become distorted, and increasing the magnification does not automatically entail an increase in optical resolution.

### New ideas

Scientific studies have shown that the brain can selectively process information from each eye individually. This gave the development engineers at Leica Microsystems a simple but ingenious idea. Why not use one beam path of the microscope for high resolution, the other for depth of field and use the ability of the brain to merge the two different images to a single, optimal spatial image. This approach, called FusionOptics™, offers two distinct advantages. Compared to existing stereomicroscopes, FusionOptics™ enables a concomitant and significant increase of resolution and focus depth. This increase in resolution can be achieved without increasing the convergence angle between the two beam paths.

### Scientific cooperation

Dr. Daniel Kiper from the Institute of Neuroinformatics at the University and ETH of Zurich worked with Leica Microsystems to design a study to corroborate the new concept. The test subjects underwent psychophysical tests to find out whether an interocular suppression takes place when both eyes are exposed to different stimuli. The test subjects observed changing arrangements of patches in various depth planes around a central fixation point (Fig. 1). Using special stereo goggles, both eyes were subjected to different stimuli. No evidence of signal suppression was observed in any of the tests. This means that the human brain is capable of using the best information from both eyes in order to compose an optimal spatial image.





## Microscopic Insights into Cellular Lipid Metabolism

# Obese and Slim Yeast Cells

Univ.-Prof. Dipl. Ing. Dr. techn. Sepp Dieter Kohlwein<sup>1</sup>

Lipids are on everyone's lips nowadays, whether  $\omega$ -3/6 fatty acids, good and bad cholesterol or just plain fat that has the annoying habit of accumulating on our hips. Serious diseases such as obesity, arteriosclerosis and type 2 diabetes mellitus are directly connected with lipid metabolism disorders. The model system yeast ("baker's yeast") provides excellent possibilities for exploring lipid-associated diseases, including the use of high-resolution microscopy.

### Fat as a risk factor

In accordance with its central importance for the organism, lipid metabolism is controlled by diverse regulation mechanisms. However, these mechanisms are overtaxed by our modern lifestyle of too much food and a lack of exercise. The dramatic development of lipid-associated diseases in industrialised countries has tended to propagate a negative image of lipid substances. Yet fat in a wide variety of forms is an indispensable ingredient of all cells. Even the much-maligned triglycerides play a vital role as a buffer for excess and potentially dangerous fatty acids in our circulation or as an energy store.

Only recently was a main fat degrading enzyme (ATGL, Adipose Triglyceride Lipase) found in fat tissue [1], demonstrating that research into fats is, now as ever, a "goldmine" for discovering new biomedically relevant key factors (see also: GOLD – Genomics of lipid-associated disorders, <http://GOLD.uni-graz.at>, a project conducted within the framework of the Austrian Genome Research program GEN-AU, and the special research project LIPOTOX, <http://lipotox.uni-graz.at>, sponsored by FWF [Austrian Science Fund]).

### A big look at small cells

Besides molecular-biological, genomic and proteomic techniques, high-resolution light microscopy is important for gaining innovative insights into cellular lipid synthesis, lipid and membrane dynamics and morphological changes in connection with lipid metabolism disorders.

With its diameter of 5–8  $\mu$ m, the yeast cell used to be considered an unsuitable specimen for light microscopic examination. Actually, the technological progress made in microscopy over the last few years through improved imaging techniques and excellent objectives can now resolve sub-cellular structures of yeast cells (Fig. 1). Confocal laser scanning microscopy offers special advantages for three-dimensional analysis of cells observed under physiological conditions over several generations [2]. Diffraction problems and stray light are minimal due to the thin cell thickness. The typical lateral and axial resolution is approx. 150 nm and 350 nm, respectively. The three-dimensional reconstruction achieved by recording a large number of "optical sections" and the simultaneous detection of several fluorophores provide completely new insights into the spatial and dynamic protein and lipid interactions in live cells.

The availability of fluorescing protein variants (e.g. GFP – green fluorescent protein) in connection with extremely simple cloning techniques has created the basis for localisation and expression studies of all proteins of the yeast proteome (of which there

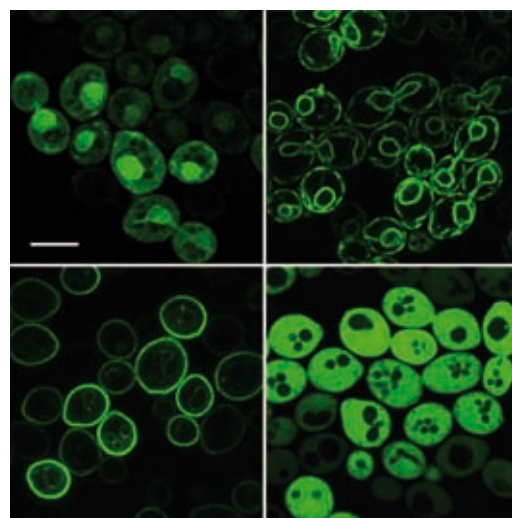


Fig. 1: Visualisation of sub-cellular structures of yeast by GFP tagging of proteins and confocal laser scanning microscopy (Leica TCS SP2 Multi-Photon System; 488 nm excitation; 500–550 nm detection; 1.4 N.A. 100x oil). From top left to bottom right: Cell nucleus, endoplasmic reticulum, plasma membrane, cytosol. Scale = 5  $\mu$ m.

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A-8010 Graz, Austria

are roughly 6,000) [3]. Robust preparation protocols for live cell microscopy and vital staining allow simultaneous observation of large cell populations, an excellent basis for collecting quantitative microscopy data.

### Through thick and thin – yeast in lipid metabolism research

Storage fats are found in the cell in the form of fat droplets. These, however, are not passive fat depots, but dynamic organelles with numerous proteins and specific biochemical functions. The biogenesis of lipid droplets is closely connected to the synthesis of the storage fats: if synthesis is switched off due to mutation, no lipid droplets are formed, and “slim” yeast cells are produced. These mutants react particularly sensitively to excess fatty acids which can no longer be incorporated into storage fat. This lipotoxicity of fatty acids is also observed in similar form in animal cells. We may therefore deduce that the synthesis of storage fat is an essential valve for rendering excess fatty acids metabolically harmless. The biogenesis and dynamics of these lipid droplets can be imaged under a high-resolution microscope by staining with vital dyes or GFP-labelled proteins (Fig. 2).

If the fat-splitting enzymes are switched off through mutation, this leads to an accumulation of triglycerides in the cell and “obese” yeast cells are produced. This defect leads to stunted growth, too, suggesting that fat degradation provides important metabolic products for cell growth [4]. The enzymes Tgl3 and Tgl4 that are involved in the fat splitting process in yeast are structurally related to ATGL and their function can be partially replaced with mouse ATGL. This finding confirms the high degree of functional and structural conservation of lipid metabolism enzymes between yeast and animal cells.

### The spatial organisation of lipid metabolism

Lipid metabolism is spread out over different areas of the cell and is subjected to a complex control process. To characterise the spatial organisation, about 600 proteins of lipid metabolism were localised as GFP fusions at high resolution [5]. Among other observations, this study has led to the identification of previously unknown proteins of the lipid droplets. Currently, all approx. 6,000 chromosomally expressed GFP fusions are being examined with the aid of confocal laser scanning microscopy and the localisation data are being made available to the scientific community in the specially designed Yeast

Protein Localisation database, YPL.db (<http://YPL.uni-graz.at>) [6] (Fig. 3). It can be clearly shown that protein localisation is not static, but is decisively influenced by the state of development of the cell and by disorders in lipid metabolism.

### Microscopy-based high-content screens

The availability of extensive collections of yeast mutants (approx. 4,500 viable deletion mutants) and suitable fluorescence dyes for lipid stores prompts the use of microscopy-based screens for lipid metabolism mutants. A recently conducted microscopy-based screen actually identified a yeast homolog of seipin that is defective in patients with Bernardinelli-Seip congenital lipodystrophy 2. The absence of this protein in yeast leads to a disturbed lipid droplet morphology [7, 8]. This implies that the identification and characterisation of preserved factors of lipid storage in yeast has tremendous potential for the understanding of the molecular causes of lipid metabolism disorders in humans.

### Future developments

The technological progress being made in microscopy plus the extensive repertoire of yeast technologies provide fascinating opportunities for understanding lipid metabolism disorders. The recently developed technique of CARS microscopy (Coherent Anti-Stokes Raman Scattering) is particularly useful for the selective detection of lipid molecules and therefore has enormous potential for imaging lipid species without the use of special fluorescence dyes. In combination with genomic and proteomic techniques, fascinating new ways of exploring lipid synthesis and dynamics in live (yeast) cells are emerging with a view to understanding lipid metabolism-associated diseases in humans.

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<http://microscopy.uni-graz.at>

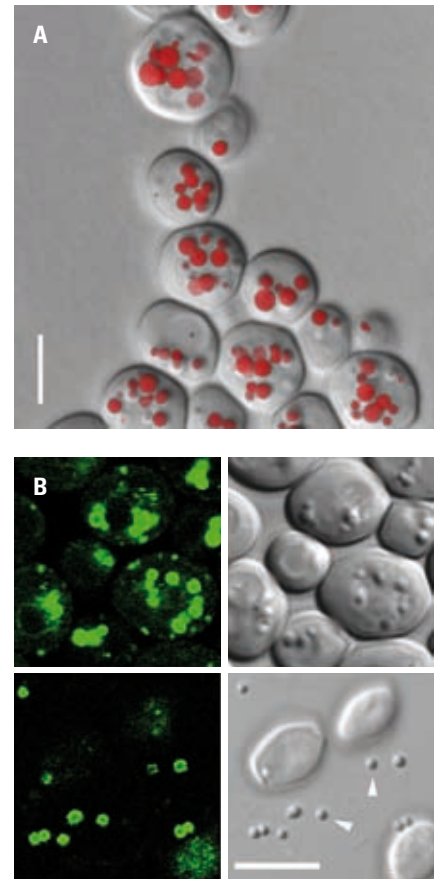


Fig. 2: A) Yeast mutants with defective triglyceride breakdown accumulate Nile Red-positive lipid droplets (LD). Scale = 5 µm. B) Visualisation of LD with the aid of GFP-labelled proteins. Left, fluorescence; Right, Differential Interference contrast. Upper row: LD in live cells, lower row: LD released from squeezed cells.



Fig. 3: Yeast protein localisation database (<http://YPL.uni-graz.at>).

## Leica Advanced Widefield Systems

# Flexible Solutions for the Diversity of Life

Markus Schechter, Leica Microsystems

Biological and medical researchers are probing deeper into the secrets of life, and widefield fluorescence microscopy is a key technology for imaging the living cell. Together with leading scientists, Leica Microsystems has further developed its successful fluorescence solutions into a modular Leica Advanced Widefield System line that can be customised to suit all live cell imaging requirements.

The new Leica Advanced Widefield System Series offers the right tool for all research needs; from routine imaging and documentation to live cell imaging workstations for complex real-time experiments. All Leica Advanced Widefield Systems utilise the same easy-to-use software platform.

### Four solutions for different application levels

The modular design of the Leica Advanced Widefield series offers the freedom to grow the system. Each of the four solutions is upgradable and can be combined with many Leica microscopes as well as many hardware and software components of Leica Microsystems.

### Design experiments with intelligent software modules ...

Common to all Leica Advanced Widefield Systems, Leica Microsystems has developed an easy-to-use, intuitive software interface that guides the user through every step of the work process. Incorporating the latest findings in ergonomic design and efficiency research, the software enables swift and effortless definition of experiment parameters and analysis. All settings are stored so experiments can easily be repeated.

Support for complex analysis procedures comes in the form of specific wizards such as the FRET Wizard. Using the Live Data Mode software module, image recording parameters can be changed without interrupting the experiment. Different jobs can be defined and combined, graphics and measurement results can be generated online. It is also possible to start experiments by incoming trigger signals or to send trigger signals to injectors or peristaltic pumps.

Fig. 1: Leica AF7000 with the inverted research microscope Leica DMI6000B, climate chamber, CO<sub>2</sub> Controller, Leica EL6000 light source and SuperZ Galvo focus.





## ... and a wide range of hardware components

Live cell experiments need optimised hardware components. Key requirements include fast and real-time image recording, stability of system parameters, particularly temperature, minimum exposure of specimens to light and highly sensitive recording techniques for weak fluorescence.

Leica Microsystems offers a wide choice of hardware components and accessories for individual configuration of research systems. The full integration of all automated functions with the software ensures perfect interaction of components in everyday use.

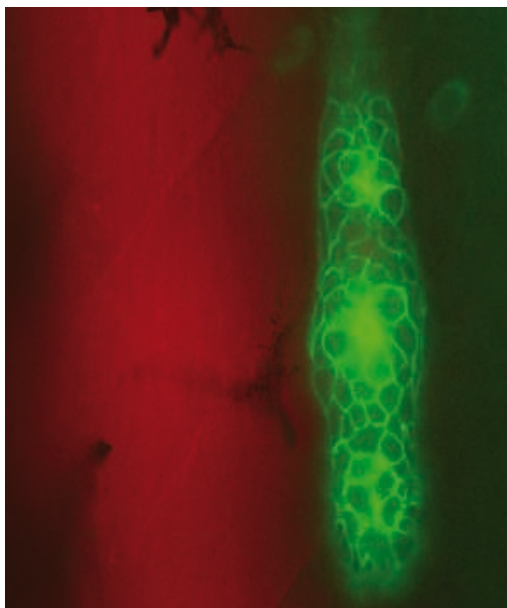


Fig. 2: Co-migration of the zebrafish mechanosensory lateral line placode (green) and innervating axons (red) at 36 hours post fertilization. Live embryo is a double transgenic carrying neuronal-specific and placode-specific constructs (NBT:dsRedExpress and claudinB:mGFP, respectively). z-stack 200 slices/80  $\mu$ m. Processing steps: dye separation and deconvolution. Courtesy of: Dr. Darren Gilmour, EMBL, Heidelberg, Germany.

### Leica AF6000 E

The entry-level software solution for high-quality fluorescence imaging and documentation. Compatible with a wide range of manual, automated and stereo microscopes and cameras from Leica Microsystems.

### Leica AF6000

The flexible system for a variety of applications in fluorescence microscopy and image analysis including time-lapse experiments and deconvolution. Compatible with automated upright, inverted, and stereo microscopes.

### Leica AF6500

The high-speed fluorescence system with all the functionality of the Leica AF6000 plus real-time controlled image acquisition for ultra fast 2D time-lapse experiments, ratio imaging, and triggering of peripheral hardware components.

### Leica AF7000

The premium solution for highly demanding applications which offers full real-time control for fast 3D time-lapse experiments, TIRF, Fura2, FRET SE, deconvolution and peripheral triggering.



Fig. 3: 5-day-old transgenic zebrafish larva; Green: GFP – actin. Courtesy of: Prof. Dr. Stephan C. F. Neuhauss, Institute for Brain Research, ETH Zurich, Switzerland.

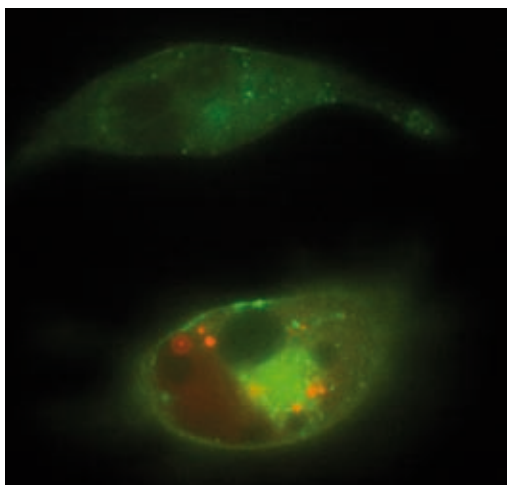


Fig. 4: Red: Galectin3-YFP; Green: Caveolin1-CFP. Courtesy of: Prof. Dr. R. Jacob, University of Marburg, Department of Clinical Cytobiology and Cytopathology, Marburg, Germany.

## Atomic Force Microscopy (AFM) and Light Microscopy

# Combined Excellence

Dr. Peter Schön, Veeco Instruments GmbH

Atomic Force Microscopy (AFM) [1] has become a powerful tool for the visualisation, probing and manipulation of biological systems from living cells down to single molecules [2]. AFM measurements can be carried out in buffer solution under physiological conditions, which is crucial for studying the structure and function of biological objects. In recent years the powerful combination of AFM with light microscopy has opened a new dimension for Life Science Research.

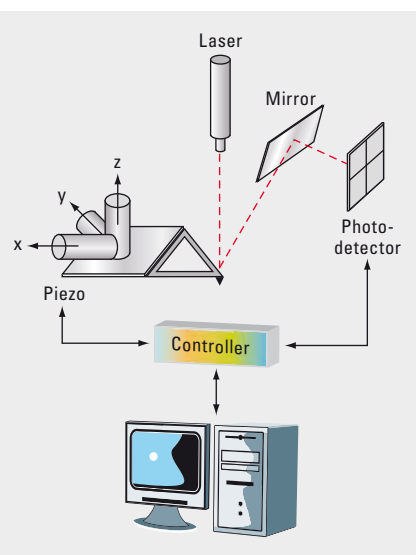


Fig. 1: The AFM principle

### How AFM works

Atomic Force Microscopy operates by scanning an ultra small tip (radius <10 nm), supported on a 100–200 µm long force-sensing cantilever, over the sample and thereby producing a three-dimensional image of the surface. Probe-sample interactions induce bending of the cantilever typically measured through a laser deflection signal change that is recorded on a photodetector. A feedback control system responds to those changes by adjusting the tip-sample distance in order to maintain a constant deflection/distance to the sample surface. It is essentially this vertical movement of the tip that translates into a topographical image of the surface with accuracy of few nm or less (Fig. 1).

Alternatively the AFM tip can be oscillated at a certain frequency thereby having only intermittent contact with the sample (TappingMode™). Lateral forces are significantly reduced in this more gentle imaging mode that has been successfully applied to image isolated and fragile objects like DNA strands or proteins inserted into lipid bilayers.

### Spatial resolution in AFM

Atomic force microscopy can provide spatial resolutions of a few nanometres and below. The actual achievable resolution depends on the size of the AFM tip (can be as small as 1 nm in radius) and the mechanical properties of the biological sample. Highest resolutions providing submolecular details have been achieved on flat and stiff (non-motile) biological membranes. It was possible to construct atomic models of supramolecular assemblies from topographical images, in the case of photosynthetic

proteins [3] or channel proteins [4]. The AFM is used to investigate structure and function of nuclear envelope surface and nuclear pore complexes during bidirectional transport of various cargos (Fig. 3). Furthermore, great progress has generally been made in cell imaging [5, 6]. On these soft and dynamic samples, structural details in the order of a tenth of a nm can be imaged.

### Force spectroscopy and force volume

The AFM can probe forces existing between AFM tip and a biological sample with high spatial resolution. For this purpose the AFM tip is moved towards the sample, contacted, indented into and retracted from

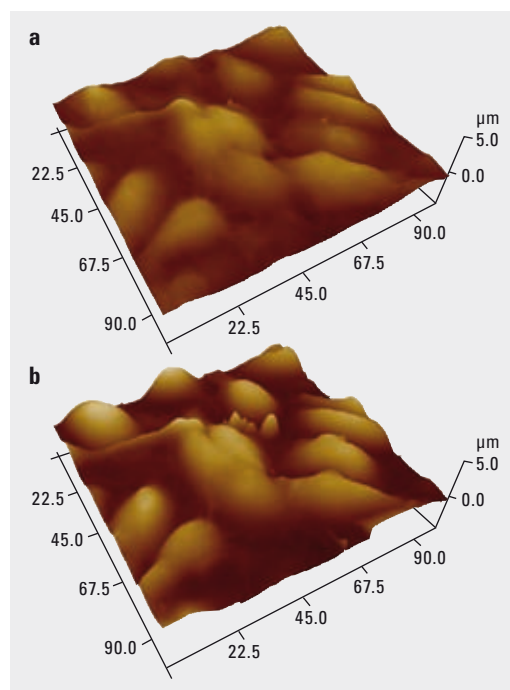


Fig. 2a–b: The two images, obtained with atomic force microscopy, show living endothelial cells exposed to either low sodium (135 mM; Fig. 2a) or high sodium (150 mM, Fig. 2b). The time period between the two surface scans is about 5 minutes. Both images contain the same cells (paired experiment). Due to the increased stiffness, induced by high sodium, cells are less flattened by the scanning tip and thus appear more prominent. Courtesy of Prof. Dr. Hans Oberleithner, M.D., Institute of Physiology II, University of Münster, Germany.

the sample. Forces induce a cantilever deflection that is recorded as a function of approach-retract travel distance. The quantification of forces is possible because the cantilever obeys Hooke's law and its spring constant can be measured. In this manner unique insight can be gained into the mechanics of a living cell and its changes upon addition of a drug for example or salt concentration change [7] (Fig.2). It was possible to distinguish cancerous cells from their healthy analogues by elasticity measurements [8].

Another striking application is related to the unzipping or unfolding of individual bio molecules like membrane proteins or DNA. Molecules can be attached between AFM tip and sample surface or even be picked or "fished" from the surface by the AFM tip [9]. Upon pulling, molecules are stretched and gradually weak intramolecular bondings are broken apart. Refolding of the molecules is possible upon approach back to the sample. Repetitive unfolding/ refolding experiments give insight into the energy landscapes of individual molecules.

The powerful Force Volume Imaging mode records an array of force curve over a selected area. Such an array gives information about the lateral distribution of mechanical properties/interactions. For example it is possible to distinguish cell elasticity between the nucleus and its softer environment.

Most interestingly, AFM tips can be modified with biological active molecules, e.g. by using a ligand modified AFM tip it is possible to screen for specific receptors on the surface of a living cell and even observe receptor trafficking [10].

### Fruitful cooperation

In recent years the powerful combination of AFM with light microscopy has opened a new dimension for Life Science Research. Veeco Instruments Inc., a leading provider of instrumentation to the nanoscience community, and Leica Microsystems have established a collaboration to drive research in the biological and nano-medical community.

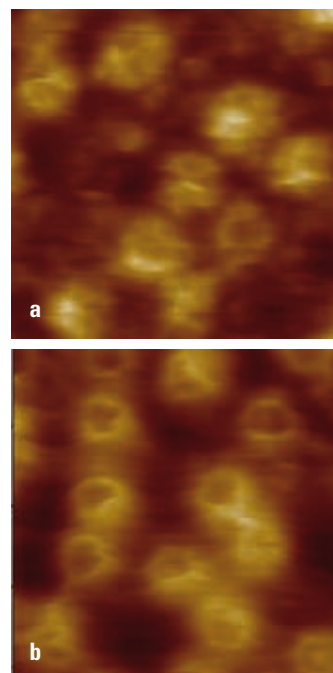
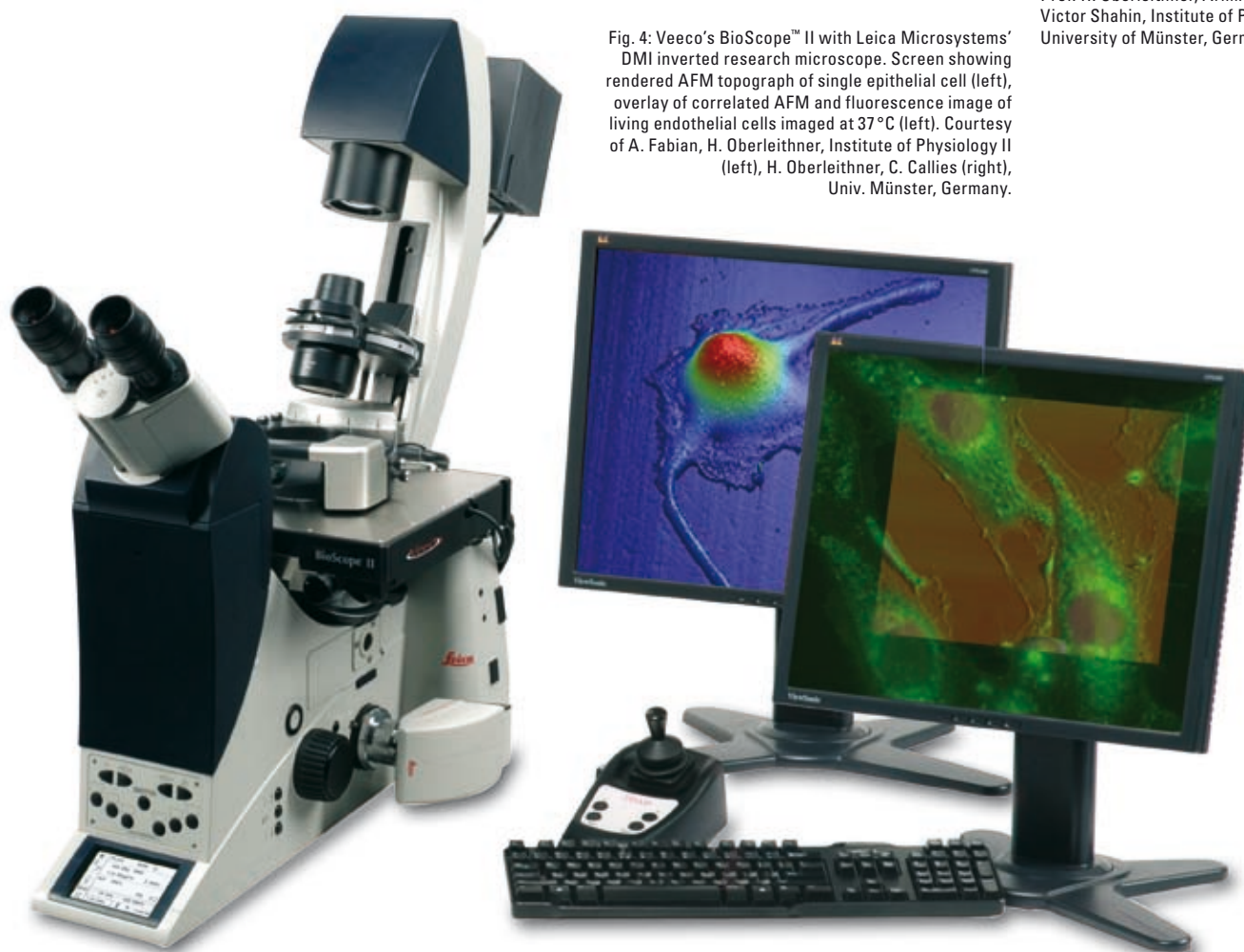


Fig.3: Studying the nuclear envelope of *X. laevis* with AFM. a: nucleoplasmatic side, b: cytoplasmatic side. Zoom into the area of  $800 \times 800$  nm. Courtesy of Prof. H. Oberleithner, Armin Kramer, Victor Shahin, Institute of Physiology II, University of Münster, Germany.

Fig. 4: Veeco's BioScope™ II with Leica Microsystems' DMI inverted research microscope. Screen showing rendered AFM topograph of single epithelial cell (left), overlay of correlated AFM and fluorescence image of living endothelial cells imaged at  $37^\circ\text{C}$  (left). Courtesy of A. Fabian, H. Oberleithner, Institute of Physiology II (left), H. Oberleithner, C. Callies (right), Univ. Münster, Germany.





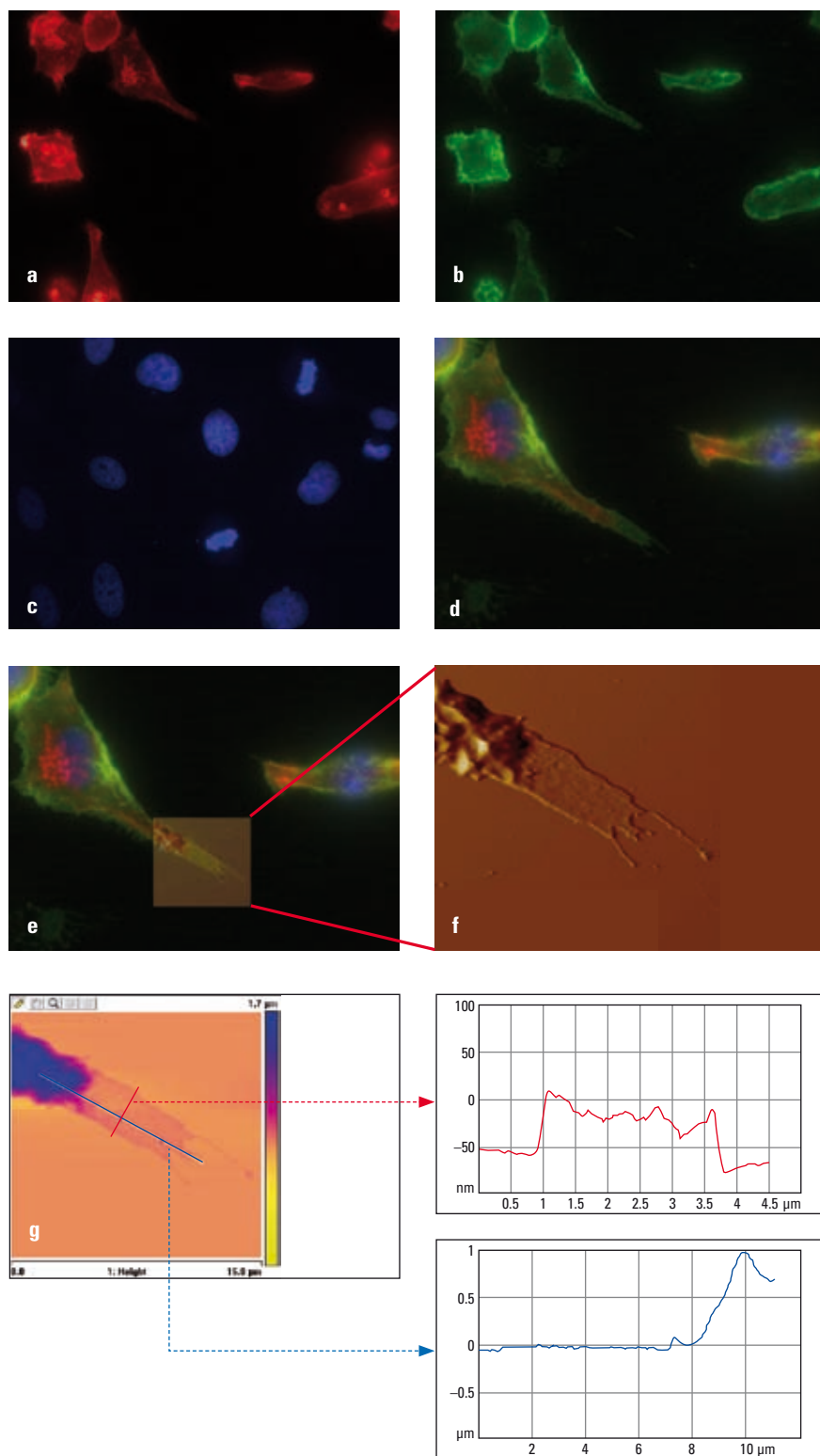


Fig. 5 a–g: Application example: Analysis of lamellipodia of moving macrophages. Correlation of structures visualised by fluorescence microscopy and AFM.

a–c: Epifluorescence

a: Red: Phalloidin – Actin

b: Green: Alexa 488 – Membrane

c: Blue: DAPI – Nucleus

d: Fluorescence overlay

e: Correlation of AFM topography with triple fluorescence labeled cells

f: AFM topography, liquid contact mode

g: Corresponding height profiles

Labelling: Triple labeling of cultured macrophages.

Courtesy of P. J. Hanley, Institute of Physiology II, University of Muenster, Germany.

The BioScope™ II has been integrated to fit into the optical path of the Leica Microsystems' DMI inverted microscopes without compromising optical functions and with the benefit of imaging, probing and manipulating biological systems – from the living cell down to single molecules. This results in a new high performance research tool, which is intuitive and easy to use (Fig. 5).

Multiple application benefits of combining optical microscopy and AFM have been demonstrated:

- Optical navigation of AFM probe to a region of interest
- Mechanical probing of elasticity, affinities and intra-molecular forces with optical identification
- Mechanical manipulation with optical observation
- Registration and overlay of optical/fluorescence images and high resolution AFM topographs

Typical biological samples are: Living and fixed cells, bacteria, viruses, tissues, membranes, lipid bilayers, filaments, single molecules (DNA, RNA, proteins), 2D and 3D protein crystals.

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More information on BioScope™ II and Veeco's AFM solutions: [www.veecolifescience.com](http://www.veecolifescience.com) or contact [bioscope@veeco.com](mailto:bioscope@veeco.com)

# Leica Scientific Forum 2008

For further information on Leica Scientific Forum events please contact Dr. Thomas Zapf: [Thomas.Zapf@leica-microsystems.com](mailto:Thomas.Zapf@leica-microsystems.com)

## September

Leica Scientific Forum France	Prof. James Pawley, University of Wisconsin, Dept. of Zoology Research, Madison WI, USA – “Biological Light Microscopy in 2020”	September 16	Institut Pasteur, CIS Auditorium, Paris
		September 17	Université de Bordeaux 2, Bordeaux
		September 18	Université Paul Cézanne Aix-Marseille, Marseille

## October

Leica Scientific Forum Heidelberg	Prof. Wendy Bickmore, MRC, Edinburgh, UK – “Nuclear organization and epigenetic mechanisms”	October 01	Communication Center of the German Cancer Research Center (DKFZ)
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## November

Leica Scientific Forum UK	Dr. Jan Ellenberg, European Molecular Biology Laboratory, EMBL, Heidelberg, Germany – “Chromatin Structure and Molecular Confinement”	November 10	Cambridge University, Wolfson Lecture Theatre, Dept. of Chemistry, Cambridge
		November 11	Liverpool University, Lecture Theatre 1, Liverpool
		November 12	Oxford University, Lecture Theatre, Dept. of Pharmacology, Oxford
		November 13	Imperial College London, Lecture Theatre 1, London

## December

Leica Scientific Forum France	Prof. Paul French, Imperial College London, UK, Department of Physics – “Fluorescence lifetime imaging (FLIM), multi-dimensional fluorescence imaging (MDFI), high resolution imaging techniques”	December 8	Institut Pasteur, CIS Auditorium, Paris
		December 9	Université de Bordeaux 2, Bordeaux
		December 10	Université Paul Cézanne Aix-Marseille, Marseille
Leica Scientific Forum Heidelberg	Prof. Dr. James G. Fujimoto, Massachusetts Institute of Technology, Cambridge, MA, USA – “Optical Coherence Tomography (OCT)”	December 12	Communication Center of the German Cancer Research Center (DKFZ)

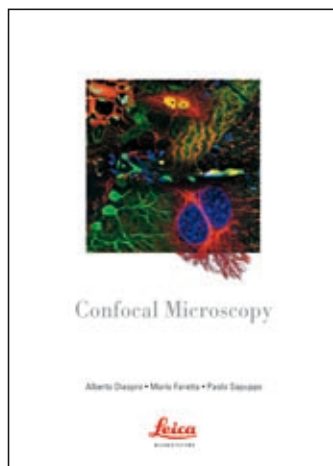
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[www.leica-microsystems.com/EU-research](http://www.leica-microsystems.com/EU-research)



The winner of our last contest: Dr. Anders Johannson, Associate Professor at Lund University, Sweden (left) won a Leica EZ4 Stereomicroscope, presented by Oliver Garner, Sales Representative Research Sweden (right).

# Events 2008

Please also visit our website on [www.leica-microsystems.com/events](http://www.leica-microsystems.com/events) for further information on Leica Research workshops in Europe.

## ELSO 2008

August 30 – September 2  
Nice, France  
<http://www.elseo.org/>

## 14<sup>th</sup> European Microscopy Congress (EMC)

September 1–5  
Aachen, Germany  
<http://www.emc2008.de>

## World Molecular Imaging Congress (WMIC) 2008

September 10–13  
Nice, France  
<http://wmicmeeting.org>

## Frontiers in Developmental Biology 2008

September 13–17  
Hyères-les-Palmiers Cedex, France  
<http://www.ibdml.univ-mrs.fr/frontiersDB/>

## MIFOBIO 2008

September 20–28  
Carqueiranne, France  
<http://mifobio2008.ibl.fr>

## Dynamic Microscopy 2008

September 22–24  
Würzburg, Germany  
<http://www.dynamicmicroscopy.de/>

## Scanlab 2008

September 23–25  
Copenhagen, Denmark  
<http://www.scanlab.nu/>

## 10<sup>th</sup> Young Scientist Meeting "Biology of Cell Division"

September 24–25  
Heidelberg, Germany  
<http://www.zellbiologie.de/>

## Salon du Laboratoire de l'Institut Pasteur (Pasteur) 2008

September 25  
Paris, France

## Notte della Ricerca 2008: "Una notte in laboratorio"

September 26  
Milano, Italy

## BIOTECHNICA

October 3–7  
Hanover, Germany  
<http://www.biotechnica.de>

## LABMED 2008

October 8–9  
Uppsala, Sweden  
<http://www.labmed2008.fi/>

## 1<sup>st</sup> Portuguese PYCHEM Meeting

October 15–17  
Lisboa, Portugal

## Midland CELLibration 2008

October 24–26  
Leicestershire/Great Britain

## 3<sup>rd</sup> Fraunhofer Life Science Symposium Leipzig 2008

October 24–25  
Leipzig, Germany  
<http://www.fs-leipzig.com/>

## Expoquimia 2008

October 24–28  
Barcelona, Spain,  
<http://www.expoquimia.com/>

## MoldMed *in vivo* Imaging "From molecule to organism"

October 27–31  
Rotterdam, Netherlands  
<http://www.molmed.nl>

## Paris ville Européenne des sciences 2008

November 12–16  
Paris, France  
<http://www.enseignementsup-recherche.gouv.fr/>

## Medica 2008

November 19–22  
Düsseldorf, Germany  
<http://www.medica.de/>

## 38<sup>th</sup> Annual Meeting of the Society for Neuroscience

November 15–19  
Washington, DC, USA

## Leica Workshops

### International Advanced Confocal Microscopy Course

September 16  
Dresden, Germany

### IV Advanced Live Cell Microscopy Workshop

October 9–11  
Madrid, Spain

### Digital Imaging

October 13–14  
Wetzlar, Germany

### Ultramicrotomy of Industrial Materials

November 24–28  
Vienna, Austria

### XI Curso Leica para el nuevo técnico-especialista en Microscopia Confocal Espectral

November 25–28  
Bellaterra, Spain

Please also visit our website on [www.leica-microsystems.com/events](http://www.leica-microsystems.com/events) for registration.

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