

RESEARCH NEWSLETTER – EUROPEAN EDITION

re SOLUTION

04 4PI MICROSCOPY

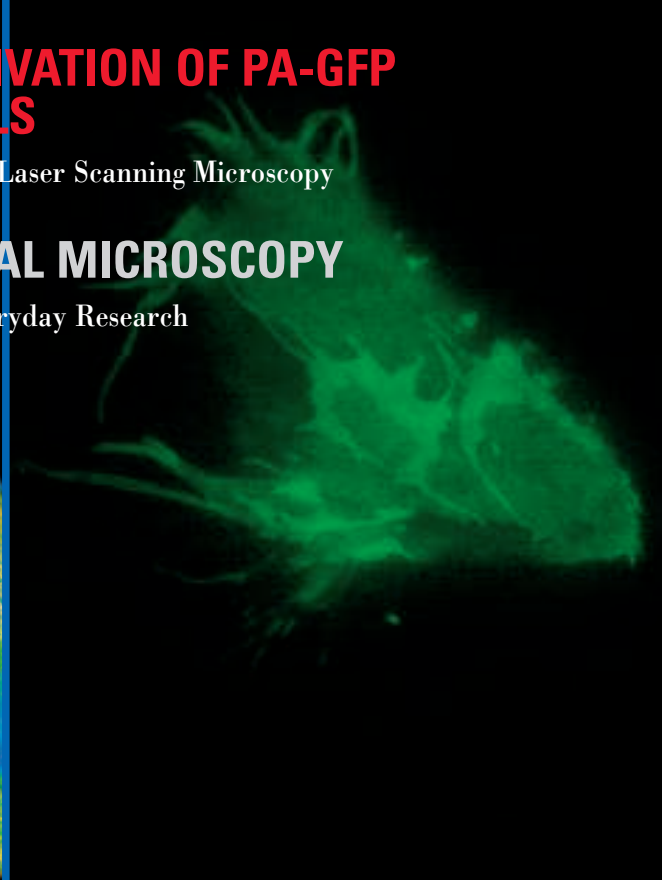
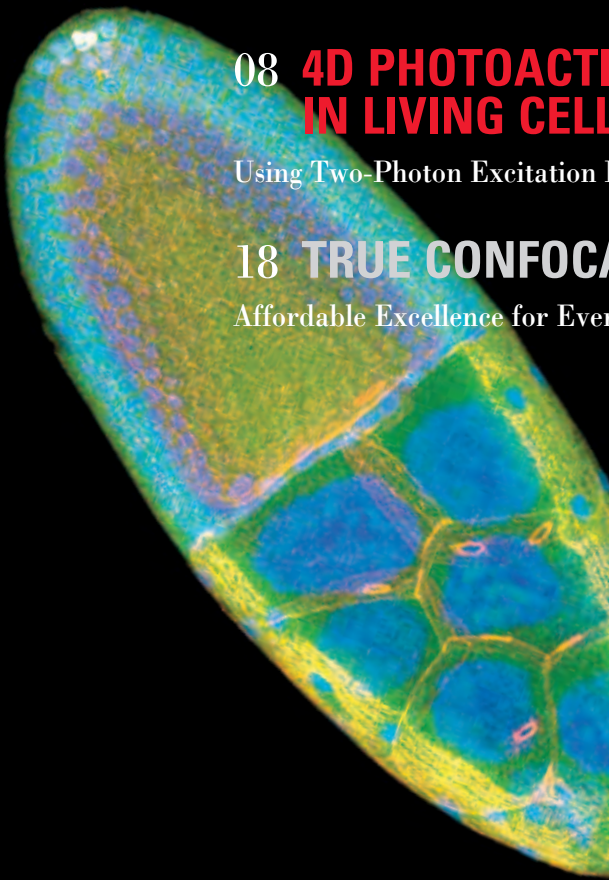
A Quantum Leap in the 3D Resolution of Fluorescence Microscopy

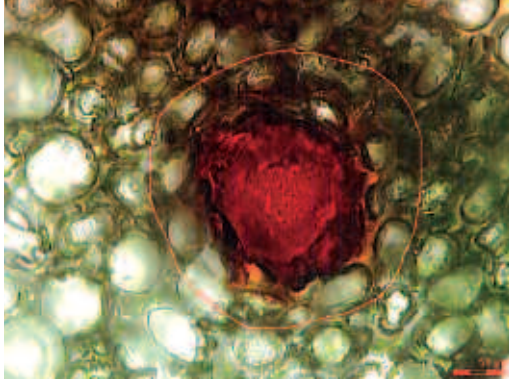
08 4D PHOTOACTIVATION OF PA-GFP IN LIVING CELLS

Using Two-Photon Excitation Laser Scanning Microscopy

18 TRUE CONFOCAL MICROSCOPY

Affordable Excellence for Everyday Research





Leica LMD6000

Whether for cancer research, other areas of pathology, proteomics, genomics, gene expression profiling or neuroscience, the newest laser microdissection system from Leica Microsystems satisfies the requirements of even the most modern research labs. The Leica LMD6000 is characterized by intelligent automation, improved performance, optimized specimen throughput and high precision, with innovations in all areas of obtaining materials for analysis. With its enhanced laser technology, the Leica LMD6000 is suitable for processing thicker specimens and harder materials, making it ideal also for botanical research applications.

Please see page 16 for further details.



Welcome to the second publication of the reSOLUTION European Research Edition!

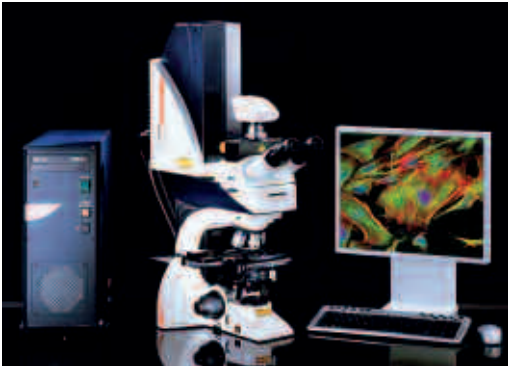
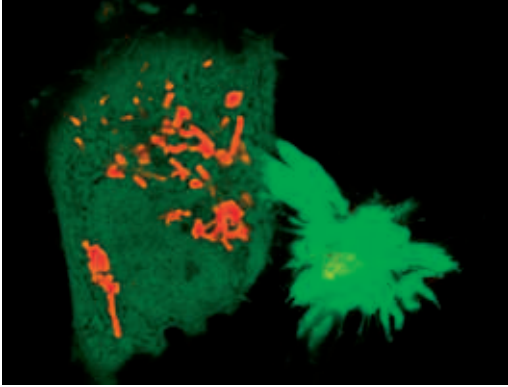
Following the track of the very first issue we want to keep on providing you with valuable information on techniques, applications, and product development in the world of Life Science Research. This issue will focus on some exciting news from the micro-world linked to new techniques like breaking barriers in terms of depth of resolution bringing near isotropic data collection in the sample with 4Pi microscopy.

Prof. Alberto Diaspro and his team will lead us in an exciting comparison of two-photon activation of the Aequorae Victoria green fluorescence protein with conventional photo activation on a Leica TCS SP2 AOBs. A study on circadian gene expression through long term observation of living cells and a report on new applications of Laser Microdissection in plant research are also included in this edition. On the instruments side our attention will be drawn to a new fully automated Leica AM TIRF system and the highest level of integration that can now bring a true spectral confocal microscope onto a desktop.

As readers as well as customers, your feedback is vital to us. Therefore we took a close look at the comments we received from all of you and are holding a new contest where you can win great prizes just by giving us your opinion.

I would like to take the opportunity to thank those of you who already collaborated to the first two issues and will be happy to receive any further contributions for the coming editions. So enjoy reading, and we look forward to hearing from you soon.

Didier Goor, European Marketing Manager Research, Leica Microsystems



Leica TCS SPE Unique in its Class of Confocal Microscopes

Leica Microsystems has released its new high-resolution spectral confocal system Leica TCS SPE. True spectral detection across the full emission range makes this system unique in its class of small and compact confocal microscopes. Optimal resolution is ensured by four solid state lasers from 488 to 635 nm, with a 405 nm option for nuclear staining. The Leica TCS SPE utilizes the brand new Leica ACS (Advanced Correction System) technology for perfect colocalization and maximum transmission. In core imaging facilities, the Leica TCS SPE serves as an expertly designed system to increase capacity and to relieve the demands on high-end confocal systems. The Leica TCS SPE is highly integrated, very compact and robust and with its small footprint and standard room requirements the system will fit into any laboratory. Its supply unit is no bigger than a standard PC and provides power, up to four lasers, an integrated PC, and a DVD-drive.

Please see page 18 for further details.

PRODUCT NEWS

4Pi Microscopy A Quantum Leap in the 3D Resolution of Fluorescence Microscopy	04
Excellent Diagnostics with a New Degree of Freedom	13
True Confocal Microscopy – Affordable Excellence for Everyday Research	18
The Way to Visualize Life's Secrets All Azimuths	20
Good Vibrations for Great Sections in Neuroscience	23

APPLICATION REPORTS

4D Photoactivation of pa-GFP in Living Cells Using Two-Photon Excitation Laser Scanning Microscopy	08
Long-Term Observation of Living Cells	14
Laser Microdissection in Plant Research – Fast, Powerful and Precise	16

EUROPEAN RESEARCH EVENTS

CONTEST Your Opinion is Valuable!	22
IMPRINT	23

CONTENTS

4Pi Microscopy – A Quantum Leap in the 3D Resolution of Fluorescence Microscopy

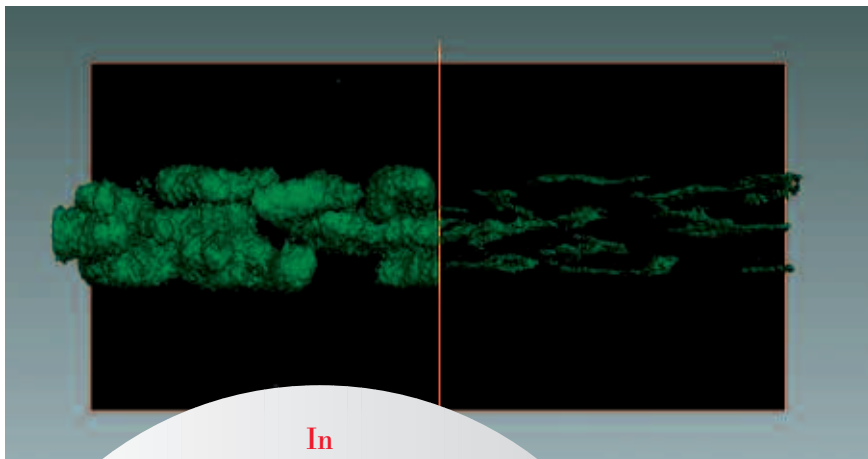


Fig. 1: Surface-rendered view of the Golgi apparatus (generated using Amira from TGS), left with "normal confocal", right with 4Pi. A Golgi resident protein, coupled with green fluorescent protein, was used as the marker (Dr. Markus Grabenbauer, EMBL Heidelberg, Germany).

In basic research, medicine and cell biology, scientists need high-resolution 3D images of structures and processes within cells to gain new research perspectives. For three-dimensional examination of living specimens, nothing but light microscopy makes sense since the only way to explore the inside of a cell without damaging it is with light. However, the resolution of a light microscope is limited, particularly perpendicular to the image plane. Prof. Stefan Hell invented a method that not only matches the depth resolution of a confocal light microscope in the image plane, but actually exceeds it. Leica Microsystems turned his idea into reality.

In 1873, Ernst Abbe recognized that the wave structure of light limits the resolution of optical methods; structures smaller than one-third of the wavelength of the light used cannot be resolved. The consequence of the diffraction limit for light microscopy is that objects in the focal plane that are less than 200 nm away from each other cannot be distinguished from one another.

They always appear as a single blurred spot in the image. The resolution along the optical axis, e.g. perpendicular to the image plane is even more problematic. Superimposed objects cannot be perceived separately if the distance between them is less than 500 nm. These limitations on light microscopy have remained largely unchanged for over 100 years.

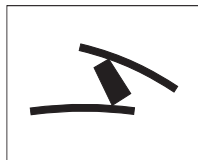
The image sharpness along the optical axis is becoming a subject of increased interest for research in bi-

ology and life sciences, since cells are transparent 3D structures. Over the last 10 to 20 years, the unstoppable trend towards 3D imaging of a cell or group of cells has also brought about the success of confocal microscopy. This type of microscopy was invented specifically to provide particular sharpness in depth. Even confocal microscopy, however, cannot provide depth resolution of better than 500 nm, which incited scientists to search for alternative solutions. Although electron microscopes provide resolution in the nanometer range, a critical disadvantage of these systems is that the specimens are exposed to hostile conditions that make it difficult to keep them alive. Electron microscopes work in a vacuum or at very low temperatures. Furthermore, only structures on the surface of the specimen are visible, so that the specimen has to be cut into very thin sections to examine it in 3D. Therefore, when viewing intact cells in their spatial depth, light microscopy is the only alternative.

Confocal microscopy with enlarged aperture

An innovative idea created a new possibility for increasing resolution in the third dimension. The focused light waves of two objectives positioned opposite each other are superimposed in such a way that they reinforce their field in the focal point, thus forming constructive interference of the opposing wave fronts in the shared focal point. The conjoined effect of the two spherical light waves, which ideally hit the focal point from all directions, results in a significantly lower focal volume. This idea, first proposed and tested by Stefan Hell, Director of the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany, turned over a new leaf in the history of light microscopy. Hell combined the aperture of two objectives of high numerical aperture, facing each other on

Winner 2005



Innovationspreis
der deutschen Wirtschaft
The World's First Innovation Award

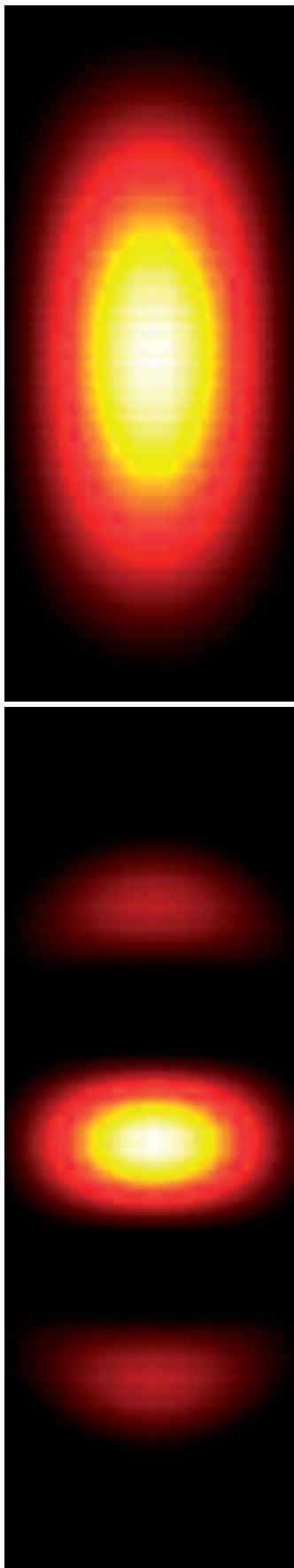


Fig. 2: Comparison of the point spread function (PSF) along the optical axis:
 – top: confocal,
 – bottom: 4Pi.

the optical axis, thus obtaining a significantly smaller focus along the optical axis as compared to the conventional confocal microscope. This new confocal technique allowed the axial resolution, which was previously limited to 500 nm, to be increased anywhere from three to five times. With this method, Hell and his colleagues attained depth resolutions below 100 nm, thus opening the door for fluorescence microscopy into the nanoworld. The name “4Pi confocal microscopy” is derived from the solid angle (4π) of a complete spherical wave.

Because the opposing objectives cannot generate a complete spherical wave, the central light spot is accompanied by two smaller sidelobes above and below the focal plane (Figure 2). In the metrological evaluation of the image data generated, the sidelobes play only a secondary role. When looking at them as a graph, they are initially obstructing; they can be eliminated, however, with a fast and simple arithmetic operation, such as linear deconvolution. The prerequisite for this is that their intensity is less than 50% of the brightness of the narrow main focus.

4Pi types A, B and C

Physically, three different variants of 4Pi microscopy are possible: type A, with 4Pi aperture in excitation, type B with 4Pi aperture in fluorescence emission, and type C with 4Pi aperture in excitation and emission.

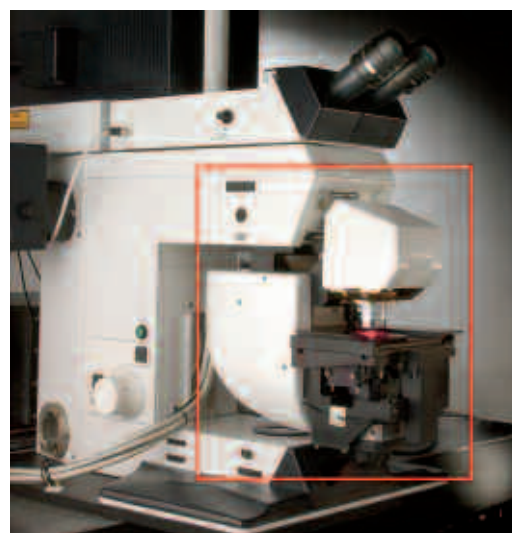
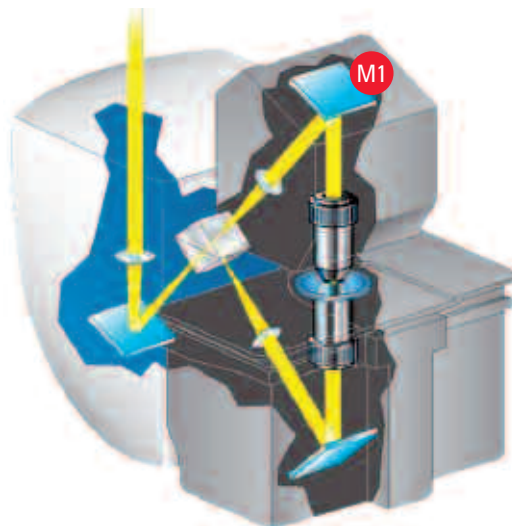


Fig. 3: Beam path in the 4Pi interferometer: The excitation beam is diverted using a mirror (M1) to the beam splitter, where it is split. A lens system and two mirrors divert the laser radiation into the objectives. Piezo drives align the bottom light paths to the top ones.

	Lateral	Axial
Resolution (according to Kino) in fluorescence:	$FWHM_{xy} = \frac{0.4 \cdot \lambda}{n \cdot \sin \alpha}$	$FWHM_z = \frac{0.4 \cdot \lambda}{n(1 - \cos \alpha)}$
For fluorescence measurements using high numerical aperture immersion objectives and an excitation wavelength of 488 nm	approx. 200 nm	approx. 500 nm
4Pi:	$FWHM_{xy} = \frac{0.4 \cdot \lambda}{n \cdot \sin \alpha}$	$FWHM_z = \frac{n \cdot \lambda}{4 \cdot n \cdot \sqrt{2}}$
4Pi type A (rough estimate (!) for 2-photon excitation with 780 nm	approx. 200 nm	approx. 110 nm

FWHM: Full width half maximum = standard unit of resolution
 α Half the aperture angle of the objective
 λ Excitation wavelength
 n Refractive index of the immersion medium
 f Correction factor (approx. 1.3–1.4)



Fig. 4: The Leica TCS 4Pi overcomes all former barriers of light microscopy.

Unlike types A and C, type B is primarily of academic interest. In its application as a method of improving resolution, type B is nearly equivalent to type A, but is more difficult to use (see information on type C below).

Combining the apertures in excitation – 4Pi type A – proved the ideal condition for technical implementation, as only one wavelength, that of the exciting laser light, must be made to interfere. When the two-photon mode of the microscope is used, sidelobes are generated that are far below the required level of 50% of the main peak and guarantee a resolution of up to 110 nm.

In 2004, Leica Microsystems introduced to the market a fluorescence microscope with a depth resolution improved by three to five times. This, the sharpest commercially available light microscope, provides 3D images with uniform resolution in all spatial directions.

Although developing a 4Pi fluorescence microscope of type C would increase the resolution to about 80–90 nm, Leica Microsystems decided against this technically more complex variant. 4Pi type C requires optimum superimposition of the entire emitted detection band, where small phase deviations can quickly lead to destructive interference. Furthermore, constructive interference in excitation sometimes combines with destructive interference in detection and vice versa. The consequence for the user is that the instrument and the gathered data are substantially more difficult to handle, which, at present, cannot always be justified by the increase in optical resolution.

Wave front synthesis in practice

In the design of the 4Pi interferometer (Figure 3) for the optical interface of a Leica TCS SP2 confocal mi-

roscope, primary emphasis was placed on the long-term stability of the system. While the upper of the two objectives is firmly anchored, the lower one is precisely positioned using piezos. Two path-folding mirrors guide two equivalent optical light beams, which are generated by a beam splitter, into the objective pair. The beam path and the mirror positions are likewise piezoelectrically controlled. The length of the axes from the beam splitter to the shared focal plane of the objective pair must be calibrated to within a couple of wavelengths. Due to the very narrow focus, the available detection volume of the Leica TCS 4Pi is also very low. Therefore, to detect even the lowest levels of fluorescence, highly sensitive avalanche photodiodes have been used in addition to the highly dynamic photomultipliers. To provide clean interference between the two wavefronts, only thin specimens (typically 25–30 μm) are used, though this is much “thicker” than a cell, which is typically 10 μm . Any phase asymmetries along the optical path can be corrected manually. Leica Microsystems has integrated the objective pair and its complex control system into a compact, user-friendly design for use in research laboratories.

Summary

4Pi microscopy provides up to a fivefold improvement in depth resolution and performance data that was technically near impossible just ten years ago. It is the first light microscopy method anywhere to provide near-isotropic resolution in 3D. The Leica TCS 4Pi microscope continues to open new avenues in basic research, such as developing effective therapies to treat diseases such as Alzheimer’s, diabetes, and malaria. Leica Microsystems won the German Business Innovation Award for the third time in 2005 for its 4Pi technology.

> TS, VM, TZ

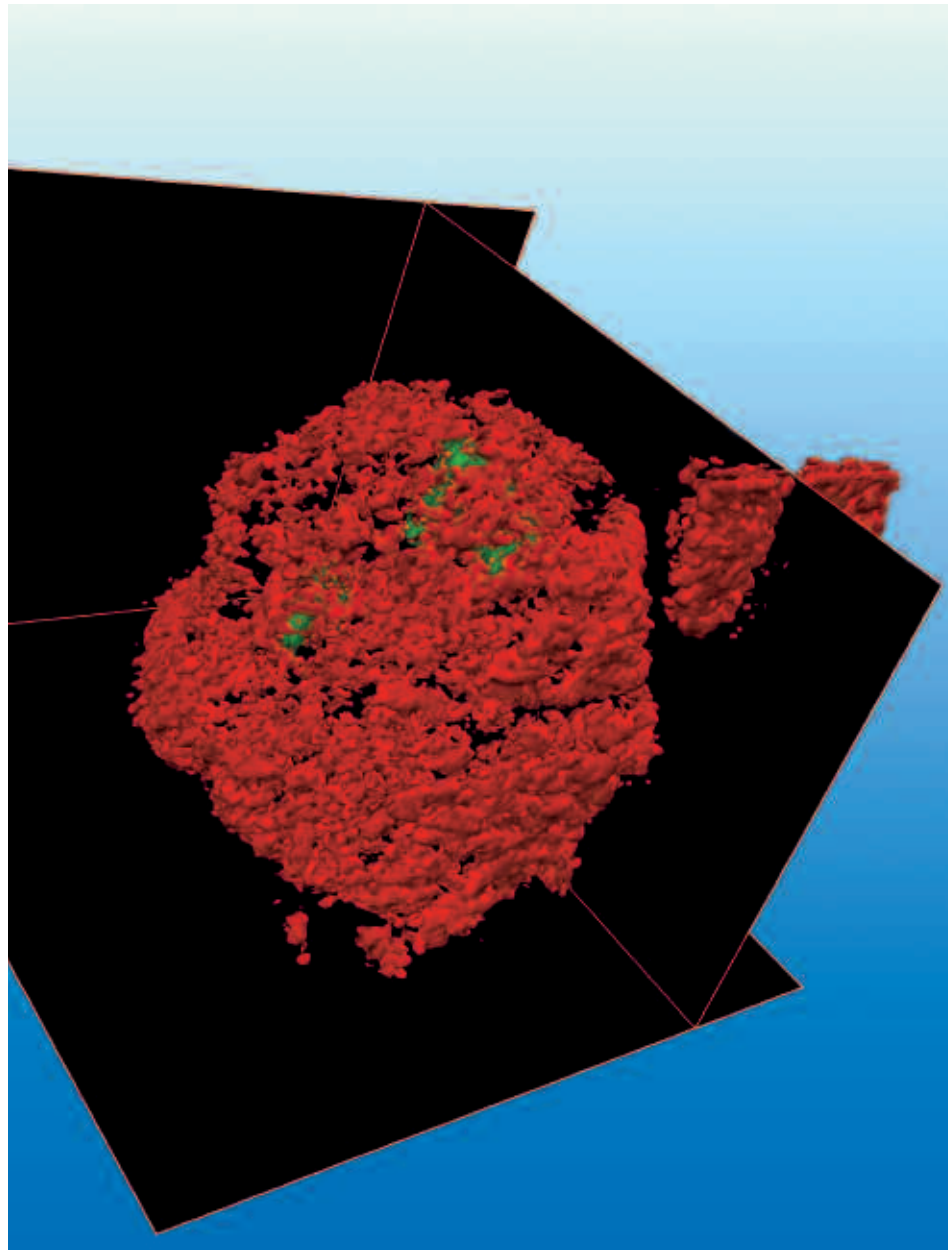


Fig. 5: Quantum dot immunostaining of Human Red Blood Cells. Human Erythrocytes labeled with anti-band-III-protein/quantum dot 1585. Surface rendered reconstruction. Diameter: approx. 8 μm , the cell is infected with plasmodia falciparum (Hoechst 33258). Courtesy Dr. James A. Dvorak and Dr. Fuyuki Tokumasu, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institute of Health, 12735 Twinbrook Parkway, Rockville, MD 20850.

References

- [1] Egner, S. Verrier, A. Goroshkov, H.-D. Söling, S. W. Hell: 4-Pi Microscopy of the Golgi Apparatus in Live Mammalian Cells. *Journal of Structural Biology* 147: 70–76 (2004).
- [2] Egner, S. W. Hell: Fluorescence Microscopy with Super-Resolved Optical Sections. *Trends in Cell Biology* 15 (No. 4): 207–215 (April 2005).
- [3] Hell, S. W.: Toward Fluorescence Nanoscopy. *Nature Biotechnology* 21 (No. 11): 1347–1355 (November 2003).
- [4] Nagorni, M., S. W. Hell: Coherent Use of Opposing Lenses for Axial Resolution Increase in Fluorescence Microscopy. I. Comparative Study of Concepts. *Journal of the Optical Society of America* 18 (No. 1): 36–48 (January 2001).
- [5] Nagorni, M., S. W. Hell: Coherent Use of Opposing Lenses for Axial Resolution Increase in Fluorescence Microscopy. II. Power and Limitation of Nonlinear Image Restoration. *Journal of the Optical Society of America* 18 (No. 1): 49–54 (January 2001).
- [6] Nagorni, M., S. W. Hell: 4Pi-Confocal Microscopy Provides Three-Dimensional Images of the Microtubule Network with 100- to 150 nm Resolution. *Journal of Structural Biology* 123: 236–247 (1998).
- [7] Schrader, M., S. W. Hell, H. T. M. van der Voort: Three-Dimensional Super-Resolution with a 4Pi-Confocal Microscope Using Image Restoration. *Journal of Applied Physics* 84 (No. 8): 4032–4041 (October 1998).

This article was published in:

Photonik International 2006
 AT-Fachverlag GmbH
 Saarlandstraße 28
 D-70734 Fellbach
 Germany

Contact: Johannes Kuppe
 Phone: +49 (0)711-952951-16
 Fax: +49 (0)711-952951-99
 kuppe@at-fachverlag.de
 www.photonik.de

4D Photoactivation of pa-GFP in Living Cells Using Two-Photon Excitation Laser Scanning Microscopy

Alberto Diaspro^{1,2}, Ilaria Testa¹, Paolo Bianchini¹, Valentina Caorsi¹, Davide Mazza¹, Giuseppe Vicidomini¹, Sara Barozzi³, Dario Parazzoli³, Pietro Transidico³, Massimiliano Garrè², Mario Faretta³

We report about two-photon activation of a photoactivatable derivative of the *Aequorea Victoria* green fluorescent protein (pa-GFP). This special form of the molecule increases its fluorescence intensity when excited by 488 nm after irradiation with high intensity light at 413 nm. Two-photon photoactivation produces an effective real three-dimensional (3D) localization of the molecular switching of pa-GFP in the bright state. Since photoactivation can be temporally actuated by the laser scanning system anytime, the combined effect produces a 4D (x-y-z-t) control of the process. Experiments were performed using fixed and living cells which expressed the pa-GFP fluorophore and microspheres whose surface was modified by specific adsorption of the unactivated fluorescent proteins. The molecular switches were activated in a range of wavelength from 720 nm to 840 nm. A comparison between the conventional activation and two-photon mode demonstrates clearly the better 3D confinement and the possibility of selection of volumes of interest within specific cellular compartments. This enables molecular trafficking studies at high signal to noise ratio.

tools offered by confocal and two-photon microscopy [9–11]. Particle tracking inside the cell largely benefits from the ability to spatially and temporally mark specific structures to follow their “signalling” over a “dark” background as made possible since the advent of pa-GFP. Pa-GFP results from a site specific mutagenesis substituting threonine 203 with histidine in wild-type GFP. This leads an excellent photo-convertible molecule producing up to a 100 fold increase in 488 nm excited fluorescence after irradiation with high energy flux at 405 nm [3]. Selective photoactivation by means of confocal microscopy immediately demonstrated that pa-GFP can be considered an optimal tool to study spatial and temporal dynamics of proteins in vivo, as tracking of lysosome and mitochondria [3, 4]. At the same time, when one is performing experiments using pa-GFP, one the first key aspects is related to the ability to perform spectral fingerprinting [12]. This is particularly useful when considering the “sea of autofluorescent molecules” present within living cells and tissues [13].

Now, in terms of spatial confinement of the photo activation process, the use of two-photon or even multi-photon excitation [14, 15] provides several favorable aspects compared to single photon confocal microscopy in photomarking biological structures to be tracked [16–18]. The highly confined excitation volumes, of the order of magnitude of subfemtoliter, due to the non-linear requirements provide a unique control of the excitation and consequently photoactivation in the 3D space.

Even though single photon confocal laser scanning microscopy can efficiently modulate excitation power in a planar sub-micron region, it fails to elicit the same control along the optical axis, being the excitation volume extended to the entire illumination cone of the objective [16]. The ability to mark specific cells in living embryos by photoactivating biomolecular markers can provide a unique tool in developmental biology studies for understanding cell fate and mechanisms of differentiation [17].

Introduction

Green fluorescent protein (GFP) from *Aequorea Victoria* [1] and its multicolored variations on the theme are among the most routinely fluorescent tracers used for biological visualization [2]. Interest has grown in more precise localization studies of protein activity and movement within a cell and we could say that a new revolution started with the advent of photoactivatable fluorescent proteins [3, 4]. Fluorescence of proteins effectively brought a “new light” in molecular and cellular biology studies [5, 6], the “fluorescence toolbox” is growing [7] and steps towards macromolecular-scale resolution, using optical microscopes, are becoming reality [8]. Within this pivotal scenario we focused on pa-GFP as photoactivatable fluorescent protein [3], and on the indispensable

¹ LAMBS-MicroScBio Research Center, Department of Physics, University of Genoa, 16146 Genoa, Italy

² IFOM, Istituto FIRC di Oncologia Molecolare, 20139 Milan, Italy

³ European Institute of Oncology, Department of Experimental Oncology, 20141 Milan, Italy

Correspondence should be addressed to Alberto Diaspro, diaspro@fisica.unige.it

4D



Fig. 1: Leica TCS SP2 workstation

Materials and methods

Confocal laser scanning microscopy

Single photon photoactivation measurements were performed on a Leica TCS SP2 confocal microscope equipped with a 63×/1.40 (OIL CS HC×PL APO) objective lens (Leica), employing the 405 nm line of a 20 mWatt laser diode. Imaging of pa-GFP pre and post-activation was obtained by the 488 nm laser line. The spectral window used to collect fluorescence spanned from 500 to 600 nm. The main channel used was channel 2.

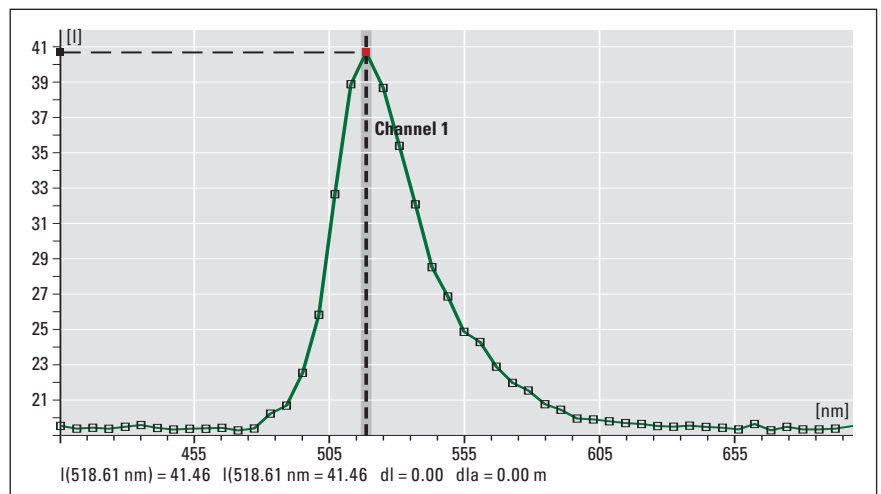
Two-photon excitation

Ti:sapphire laser source was directly coupled into the scanning head of a Leica TCS SP2 AOBs confocal microscope through the infrared port, figure 1. Measurements were collected using an average power of $\langle P \rangle_{\min} = 2.5$ mW up to $\langle P \rangle_{\max} = 12.5$ mW at the focal plane for two photon induced photoactivation. Imaging of the activated protein was obtained using the 488-nm line of a 20-mW Argon ion laser. Images were collected using a 100 x/1.4 (OIL HCX PL APO) objective. The two-photon activation process followed a previously established procedure [16]: it was first primed by focusing a pulsed infrared laser beam on a region of the sample with $\lambda = 750$ nm; subsequently, the unzoomed area was excited with $\lambda = 488$ nm and $\langle P \rangle = 0.04$ mW (as measured at the objective back plane) to visualize the activated proteins. The spectral window used for collecting fluorescence was 500–600 nm, channel 2. AOBs and spectral unit were used for proper checking of the protein emission spectra before and after the photoactivation process, figure 2.

Samples

Phoenix and HeLa cells were grown in standard culture conditions at 37 °C, 5% CO₂ in DMEM medium supplemented with 10% North American Fetal Bovine Serum (Gibco Europe, Paisley, UK). pa-GFP was a generous gift from Dr. George Patterson. HeLa transient transfection was performed using FuGene (Boehringer-Ingelheim Italia S.p.A., Milan, Italy) reagent according to manufacturer instructions. Cells were harvested and fixed after 48 hours. Before mounting, cover slips were stained with the DNA dye TOPRO 3 (Molecular Probes Europe, Leiden, Netherlands) to facilitate cell identification. Phoenix cells

Fig. 2: Emission spectrum of expressed pa-GFP in living cells (a) following two-photon photoactivation (b) as tracked using the spectral scouting system (c) [16].



were transfected with pa-GFP encoding DNA using Calcium Phosphate. After 24 hours, cells were lysed in cold 1X PLC buffer (50 mM HEPES, 1.25% glycerol, 150 mM NaCl, 166 mM $MgCl_2$, 1% TRITONX-100, 0.001% EGTA, 10 mM Napyruvate, 0.1 mM NaOrtovanadate, 0.01 mM PMSF, Aprotinin, pepstatin and leupeptin) on ice for 1 hour. Cell lysates were incubated with anti GFP polyclonal antibody and subsequently with Protein A sepharose beads, used as phantom samples. For imaging, beads were resuspended in a 90% glycerol solution containing diazabicyclo-(2.2.2) octane antifade (Sigma-Aldrich S.r.l., Milan, Italy). The modified spheres were sandwiched between ethanol/acetone cleaned cover slip and glass slide. To avoid drying, the sample was sealed with ordinary nail polish [16].

Results and discussion

Two-photon activation, similarly to two-photon excitation, is expected to be a three-dimensional confined process. This can be proved by imaging selected volume of interest within artificial samples containing the pa-GFP or living cells able to express the protein. We checked the photoactivation properties as a function of activation wavelength and power [16] using phantom samples made by pa-GFP immobilized on beads. Green fluorescence was initially excited at 488 nm to visualize the pre-activation intensity. The activation process was then primed by focusing a pulsed infrared laser beam on a $22 \mu m^2$ region (512×512 pixel). For the activation process the dwell time per pixel was varied between $4.88 \mu s$ and several milliseconds (ms). Subsequently, the unzoomed area was imaged with the acquisition parameters used before the activation process.

Fig. 3: Irradiation time versus photoactivation efficiency.

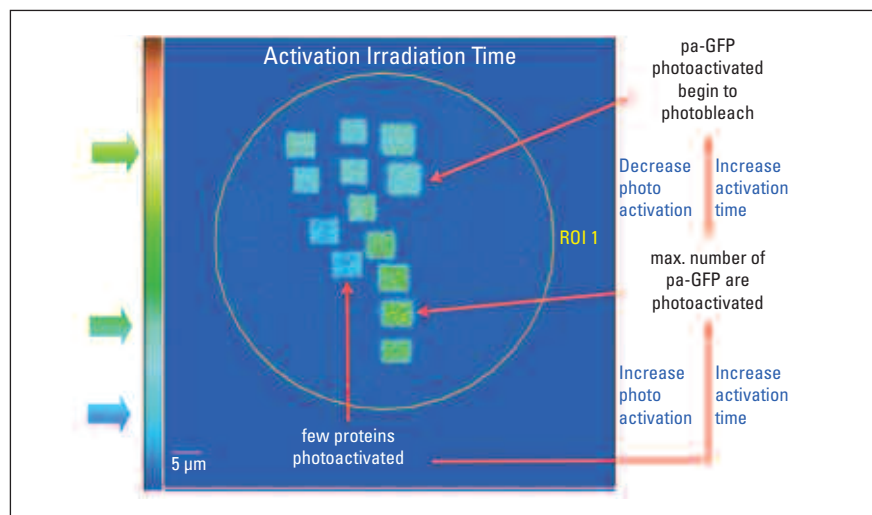


Figure 3 shows different areas photoactivated under different conditions on the surface of the pa-GFP-

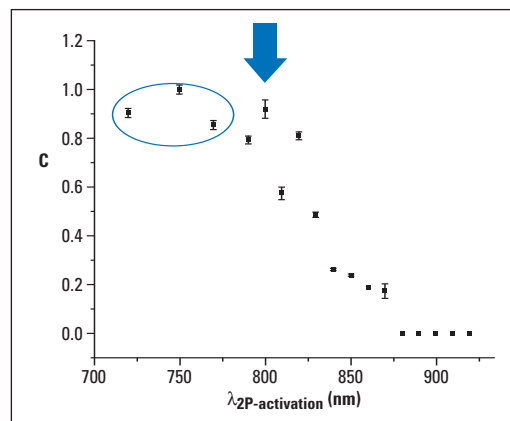


Fig. 4: Displayed data are normalized to the value obtained at 750 nm for each measurement. The error bars represent the standard error for the distribution of the mean value which were obtained from several independent measurements [16].

sphere used as phantom. The images before and after activation were analyzed for determination of mean intensity values. The ratio (C) of mean fluorescence intensities of the activated areas was taken as a measure for the efficiency of the photoconversion process as function of the photo-activation wavelength, figure 4.

Due to the locally restricted non-linear excitation probability, activated volumes of finite thickness were expected in dependence on the provided laser light intensity [14, 15], whereas single photon induced activation should extend along the whole beam path of the light in the sample. A comparison between the one-photon ($\lambda = 405$ nm) and two-photon activation process is shown in Figure 5. The thickness of the two-photon activated volume is limited to a narrow region inside the cell, while one-photon activation results in a fully activated cell in axial direction.

The intensity profile $I(x, y, z)$ of a photoactivated volume within the nucleus is shown in figure 6. The intensity profile is also a function of the power being used. The change of the activated volume is in accordance with the expectation based on the change in the intensity distribution $I(z)$ that follows an inverse fourth power law as a function of the distance, z , from the geometrical focus of the lens. Using parameters like the excitation wavelength, the numerical aperture, the refractive index of the sample, it is possible to predict an arbitrary intensity value in dependence on the distance from the focal plane [16].

The intensity of the fluorescence emitted after photoactivation represents the edge between activated and not-activated volume and can therefore be considered as a threshold indicator for determining the activation process parameters. The thickness of the activated volume increased as a function of the laser

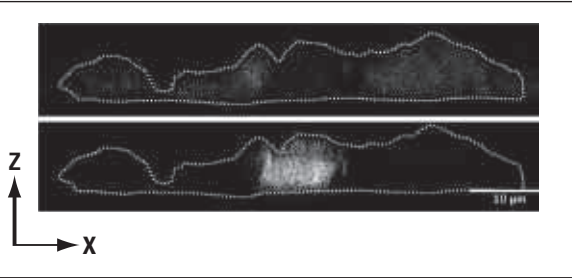


Fig. 5: Photoactivation extension along the z-axis using two-photon (above) and conventional (below) photoactivation of pa-GFP within a living cell (shape is contoured by white lines).

light energy. The values for different volumes of interest can be summarized using the following data triplets reporting scan time per pixel [μs], activation power [mW] and FWHM(z) [μm], namely: (19.6, 5, 2.35); (4.9, 17, 3); (9.8, 10, 3.5).

To quantify the error that can be made due to the location on the sphere, of different areas, covering the whole sphere, with the same settings were activated. The difference in the fluorescence intensity after irradiation with 488 nm was found to be 10%. This uncertainty of the measured intensity values is intrinsic and will not be displayed additionally to the statistical errors. To identify the best working efficiency a full activation spectrum would be useful. With our set-up, a range of wavelength between 720 nm and 920 nm is

accessible which covers very well the known absorption cross sections of wild-type GFP and at least the absorption peak of EGFP. For the acquisition of the spectrum the different probability of absorbing two photons in dependence on the wavelength was taken into consideration (power correction for higher wavelengths).

To measure the success or efficiency of the activation process the ratio of the fluorescence intensities (irradiation with $\lambda = 488$ nm) after two-photon activation and before was considered. It was found that the conversion efficiency drops dramatically for wavelengths above 830 nm (for high powers and long times it is nevertheless possible) [16]. Further, the efficiency shows a high level for wavelengths between 720 nm and 750 nm. Up to 800 nm another efficiency level applies. Unfortunately, the following values show strong fluctuations, no neither a clear assignment to the former level, nor an assignment to the low level efficiency shall be made at this point without further investigations.

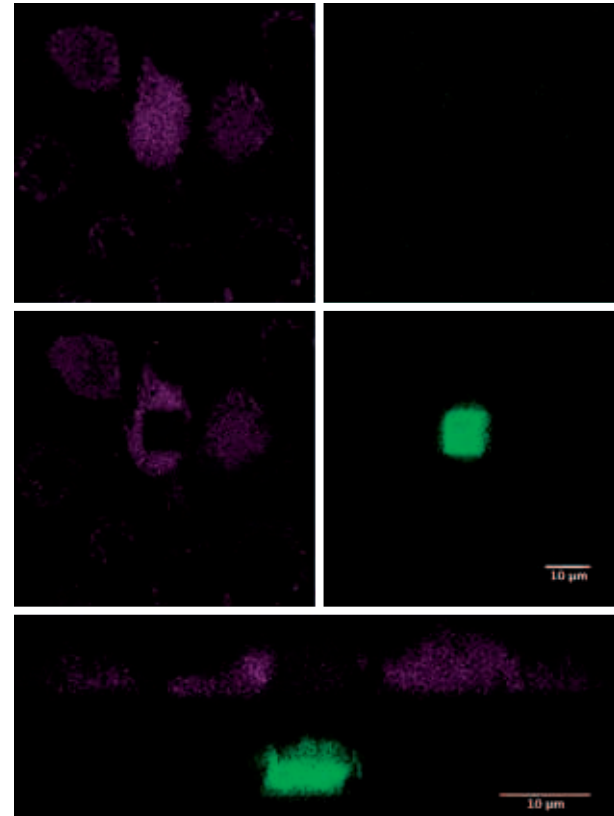
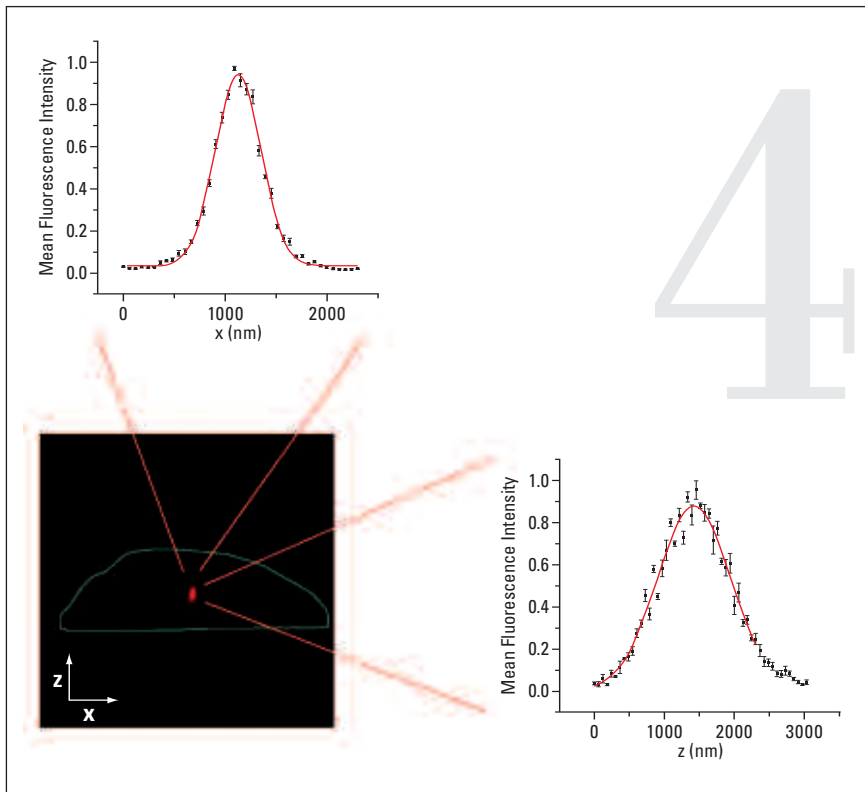


Fig. 7: Projection of optically sectioned cells, obtained with Leica TCS SP2 AOBs confocal microscope: in violet 405 nm low intensity excitation (not activated pa-GFP), in green 488 nm excitation (activated pa-GFP) of wt-pa-GFP transfected HeLa cells. First row – not all activated; second row – after photoactivation of a portion that results in a black rectangle in the non-activated column (violet) and bright green in the photoactivated; third row – post photoactivation x-z section.

Fig. 6: HeLa cells expressing H2B-pa-GFP were photoactivated employing a 750 nm wavelength. Photoactivation has been obtained parking the beam on a single point in the field of view for different activation times (1: 1 second; 2: 3 seconds) to study spatial confinement. Spatial extension in the focal plane providing a full width half maximum (FWHM) of the activation profile around 400 nm and an extension along the optical axis around 1 μm , close to the theoretical resolution limit provided by two-photon microscopy.

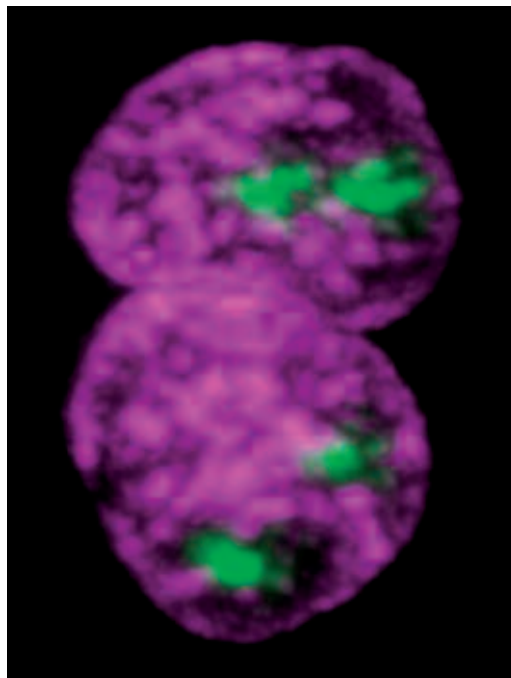


Fig. 8: Projection of optically sectioned nuclei, obtained with Leica TCS SP2 AOBs confocal microscope: in violet 405 nm low intensity excitation (not activated pa-GFP), in green 488 nm excitation (activated pa-GFP) of pa-GFP-histone H2B transfected HeLa cells.

Conclusion

In the present work, the photophysical properties of pa-GFP under multiphoton excitation have been outlined showing the possibility to employ two-photon microscopy in combination with photoactivatable markers for protein dynamic studies. Pa-GFP exhibited the same photoconversion properties when excited in far red wavelength range using two-photon excitation in comparison with photoactivation at 405 nm: the absorption of photons can induce an increase of absorption cross section at 488 nm. This effect can be efficiently employed to mark submicron regions in the whole cell selecting, case by case, the appropriate volume of interest. Examination of the activated spatial volume as a function of the excitation energy showed that intensity modulation can be efficiently used to induce spatially controlled protein photoconversion along the optical axis providing a unique possibility to dynamically identify single 3D structures and considering final 4D (x-y-z-t) processes.

Fig. 9: Prof. Albert Diaspro (3rd from left) and his research group.



Two-photon activation can be efficiently used to track the fate of proteins and other biological macromolecules in living cells following cell cycle steps, and applications in terms of 3D memories could also be pursued. It is worth noting that coupling the selective expression of a protein within biological samples with the high localization level of non-linear processes produces a formidable tool for scientific research. Moreover, the exploitation of non linear processes involved in the interaction between light and proteins can lead to macromolecular resolution levels while keeping the advantages of using optical microscopy [8, 19, 20].

> AD

Acknowledgements

Authors are indebted to George Patterson and Jennifer Lippincott-Schwartz for pa-GFP availability.

References

- [1] Shimomura, O.: The discovery of aequorin and green fluorescent protein. *J. Microscopy* 217: 3 (2005).
- [2] Tsien, R. Y.: The Green Fluorescent Protein. *Annual Review of Biochemistry* 6: 509 (1998).
- [3] Patterson, G. H., J. Lippincott-Schwartz: A Photoactivatable GFP for Selective Photolabeling of Proteins and Cells. *Science* 297: 1873 (2002).
- [4] Patterson, G. H., J. Lippincott-Schwartz: Selective photolabeling of proteins using photo activatable GFP. *Methods* 32: 445 (2004).
- [5] Miyawaki, A.: Fluorescent proteins in a new light. *Nat. Biotechnol.* 22: 1374 (2004).
- [6] Diaspro, A.: Shine on ... proteins. *Microsc. Res. Tech.* 69: 149 (2006).
- [7] Giepmans, B. N., S. R. Adams, M. H. Ellisman, R. Y. Tsien: The fluorescent toolbox for assessing protein location and function. *Science* 312: 217 (2006).
- [8] Donnert, G., J. Keller, R. Medda, M. A. Andrei, S. O. Rizzoli, R. Luhrmann, R. Jahn, C. Eggeling, S. W. Hell: Macromolecular-scale resolution in biological fluorescence microscopy. *Proc. Natl. Acad. Sci. USA* 103: 11440 (2006).
- [9] Pawley, J. (ed.): *Handbook of Biological Confocal Microscopy*. Plenum (2006).
- [10] Diaspro, A. (ed.): *Confocal and Two-Photon Microscopy: Foundations, Applications, and Advances*. Wiley-Liss (2001).
- [11] Yuste, R.: Fluorescence microscopy today. *Nature Methods* 2: 902 (2005).
- [12] Patterson, G., R. N. Day, D. Piston: Fluorescent protein spectra. *J. Cell Sci.* 114: 837 (2001).
- [13] Piston, D. W., G. H. Patterson, S. M. Knobel: Quantitative imaging of the green fluorescent protein (GFP). *Methods Cell Biol.* 58: 31 (1999).
- [14] Diaspro, A., G. Chirico, M. Collini: Two-photon fluorescence excitation and related techniques in biological microscopy. *Quarterly Reviews of Biophysics* 38: 97 (2005).
- [15] Diaspro, A., P. Bianchini, G. Vicidomini, M. Faretta, P. Ramoino, C. Usai: Multi-photon excitation microscopy. *Biomed. Eng. Online* 5: 36 (2006) – <http://www.biomedical-engineering-online.com/content/5/1/36>.
- [16] Schneider, M., S. Barozzi, I. Testa, M. Faretta, A. Diaspro: Two-photon activation and excitation properties of PA-GFP in the 720-920-nm region. *Biophys. J.* 89: 1346 (2005).
- [17] Post, J. N., K. A. Lidke, B. Rieger, D. J. Arndt-Jovin: Photoactivation of PAGFP in live *Drosophila* embryos: two-photon activation and phototoxicity. *FEBS Letters* 579: 325 (2005).
- [18] Chen, Y., P. J. MacDonald, J. P. Skinner, G. H. Patterson, J. D. Muller: Probing nucleocytoplasmic transport by two-photon activation of PA-GFP. *Microsc. Res. Tech.* 69: 220 (2006).
- [19] Diaspro, A.: *New World Microscopy*. *IEEE Engineering In Medicine And Biology Magazine* 15: 29 (1996).
- [20] Willig, K. I., R. R. Kellner, R. Medda, B. Hein, S. Jakobs, S. W. Hell: Nanoscale resolution in GFP-based microscopy. *Nature Methods* 3: 721 (2006).

Leica DMD108

Excellent Diagnostics with a New Degree of Freedom

In microscopic diagnostics, easy to use instruments are as essential as brilliant images. Leica Microsystems is continuously working on innovative developments in order to constantly improve the optical quality and precision of microscopic imaging techniques. Ergonomics is also a key consideration for integrating microscopic processes into intelligent and time saving solutions. For the increasing demands in microstructure analysis, digital devices are in many respects superior to conventional microscopes.

Leica Microsystems has designed a new network imaging solution that addresses not only the growing workload in today's busy laboratory but also the need to quickly share data. The innovative Leica DMD108 increases physical comfort, significantly speeds daily workflow without changing the process, and provides an easy-to-use network solution for sharing data. The solution has been extensively tested with users to ensure that their daily requirements are met.



Fig. 1: The new network imaging solution Leica DMD108 increases physical comfort and significantly speeds daily workflow.

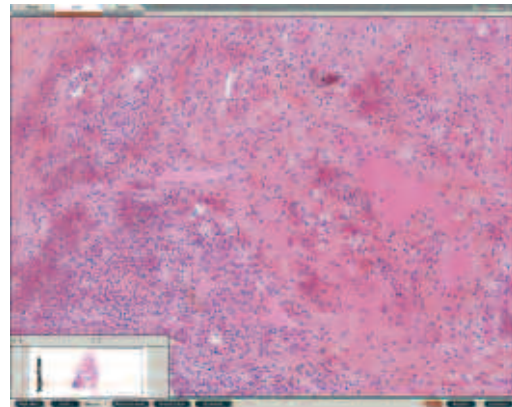


Fig. 2: Macro generator with an easy-to-use navigation function.

The user no longer needs to look through a microscope's eyepieces to view and analyze specimens. This results in a truly ergonomic working position. The system provides Leica's renowned, high-quality images directly on a monitor using a high-resolution camera and powerful image processing software. The Leica DMD108 generates high-resolution images with brilliant color fidelity that equal those produced using a conventional microscope.

With the Leica DMD108 the user can photograph specimen details of interest or compare tissue sections. Images are easily stored with the click of a button and can be retrieved at any time. Size ratios are also calculated by a simple keystroke. The user can then audio-record the diagnosis directly onto the system, and the diagnosis is ready for transmission.

The Leica DMD108 provides an ideal link between pathologists around the world. Now, holding live discussions about specific specimens is amazingly easy with Leica's new network imaging solution, and it is far more convenient than conventional discussion microscopes. A second monitor or high-resolution data projector can be added to the system for training, conferences, and discussions. Images can even be emailed during the work process; whenever a second opinion is required from a colleague who is not linked to the network.

> AS

Long-term Observation of Living Cells

For up to three days, living cells undergo continuous observation in order to study circadian gene expression in the individual cell. In series of experiments such as these, it is particularly important that the focus remains stable. Other requirements include a very short exposure time, optimum CO₂ values and humidification of the climate chamber. Only under these conditions is it possible to carry out such long experiments with living cells.

ology is based on negative feedback loops, in which proteins are synthesized and automatically regulate their own deactivation in a continuous 24-hour rhythm. Flying between different time zones is one circumstance that upsets circadian rhythms. In this case, the result is jet lag, which leads to typical symptoms that include difficulty in falling and staying asleep and digestive disturbances.

In the 1990s, a research group headed by Professor Ueli Schibler was able to prove the existence of circadian rhythms in fibroblastic cell lines [1]. To this end, cells were subjected to shock by using greatly altered serum concentration. Then, in regular intervals, the concentration of messenger RNA of certain proteins was measured and quantified. Based on these experiments, the research group was able to prove that the synthesis of these proteins in fibroblastic cell lines follows a 24-hour rhythm. However, the experiments were not able to prove that the rhythms are continuous. Whether circadian rhythms exist at all times or are triggered directly by the shock was left unanswered. Only single-cell experiments could provide the answers that were sought. Schibler's workgroup at the University of Geneva carried out the experiments for rhythmic protein synthesis and deactivation on the single-cell level using the Leica AF6000 LX fluorescence workstation. This microscopy system was specially designed for long-term experiment series and provides the ideal conditions for living cells. As the results obtained by the research team confirm, long-term observation of living cells will continue to be an important aspect of modern biomedical research.

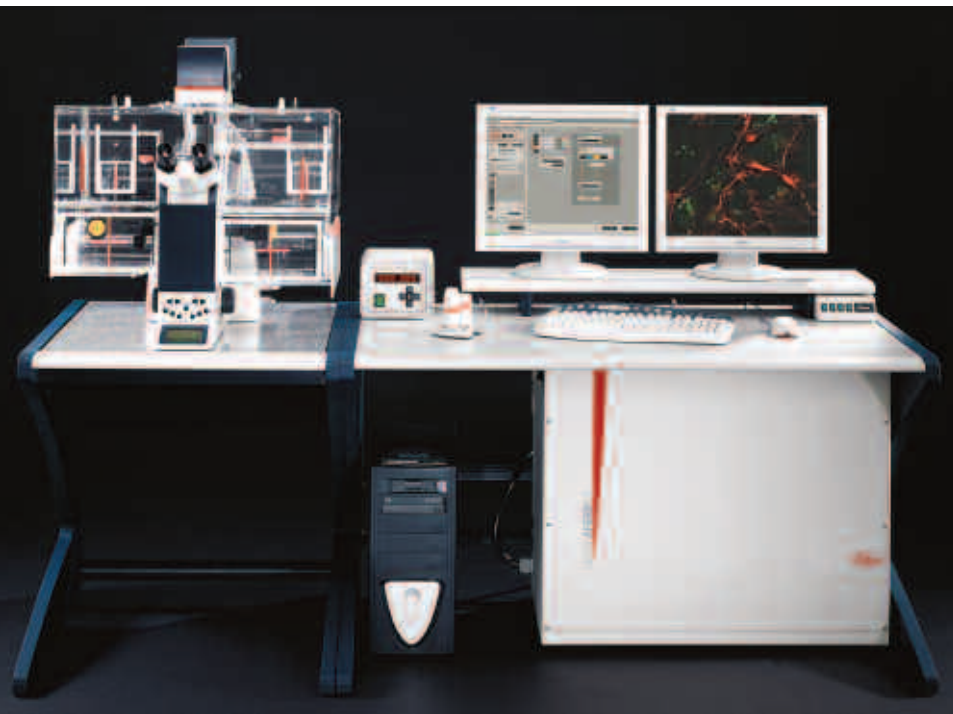


Fig. 1: The Leica AF6000 LX fluorescence workstation prevents thermal effects, thus eliminating instability of the focus – particularly important for time-lapse photography of living cells.

Heartbeat, blood pressure, the sleep/wake cycle, body temperature, kidney activity, digestive tract, hormonal changes and libido: all of these are controlled by circadian rhythms. Suprachiasmatic neurons in the brain are the control units for the body's circadian rhythms in mammals, including human beings. Light affects the function of the central clock located in the brain, which, in turn, controls all of the peripheral clocks in the organs. This rhythmic physi-

Examinations on the single-cell level

To examine circadian gene expressions in the single cell, Schibler formed a research team with Emi Nagoshi, Camille Saini, Christoph Bauer, Thierry Laroche and Felix Naef. Based at the University of Geneva, the workgroup studied the rhythmic protein synthesis and deactivation on a single-cell level [2]. They examined the living cells for up to three days at a time using the Leica AF6000 LX fluorescence work-

station. This multidimensional image analysis system consists of the Leica DMI6000 B inverted research microscope, other hardware components, application software and a selection of camera systems. For their examinations, the scientists tagged the cell cultures with Venus, a tag that is derived from YFP and has particularly intense fluorescence. A PEST1 sequence ensures that the proteins are broken down very quickly. Based on the synthesis rates of the proteins, it is possible to determine whether circadian rhythms exist in single cells. The profiles of the yellow fluorescent cell nuclei indicate whether there is indeed a periodicity between deactivation and synthesis – similar to the circadian rhythms in the brain and organs. During the single-cell examinations, the scientists observed both untreated cell populations and populations that were subjected to a shock with greatly changed serum concentration.

Stability is critical for successful experiments

The basic prerequisite for long-term exposures – such as those carried out at the University of Geneva – is a highly stable system. The cells are kept under long-term observation for up to three days, and it is critical that the focus does not drift over this period. The Stability Manager of the Leica DMI6000 B, developed specially for long-term experiments, prevents this drift. The inverted research microscope with the climate chamber allows optimal temperature control. This prevents thermal effects that can cause the focus to become unstable. To eliminate phototoxicity and bleaching of the cells, the lighting intensity must be kept low and the exposure time kept as short as possible while the images are captured. A feature that was especially important to the Geneva research group was the AF6000 LX's ability to capture images in real time, whereby the shutter and illumination can be controlled with millisecond precision. This optimum synchronization ensures that illumination takes place only when images are being captured. An incubator provides ideal conditions for capturing images of living cells over long periods. The climate chamber of the Leica AF6000 LX allows levels of CO₂ and humidification to be controlled so that the cells are exposed to conditions identical to those of an incubator. The researchers at the University of Geneva were finally able to prove the continuous existence of circadian rhythms in single cells. They proved that the cycle between protein synthesis and deactivation in the cells is permanent; the rhythms do not merely result from the shock caused by the greatly changed serum concentration. The shock synchronizes the cells, with the result that the fluorescence profiles of the cell nuclei adapt for a certain period to the event (Figure 2a). In untreated cells, the profiles are not

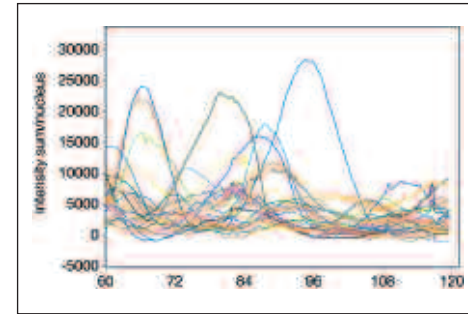
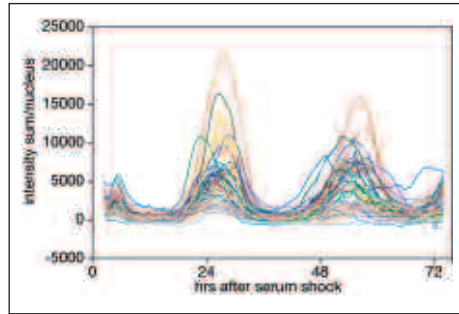


Fig. 2: Fluorescence profiles of the cell nuclei after the shock (2a, left) and of cell nuclei without changed serum concentration treatment (2b, right).

synchronous (Figure 2b), but a rhythm is nonetheless present. Furthermore, Schibler's research team was able to prove that, during cell division, the constant periodicity is passed on from the mother to the daughter cell. Only by using the capabilities of the Leica AF6000 LX fluorescence workstation was the team able to obtain these conclusive results. The system's stability allowed accurate and easy time-lapse photography of living cells.

Future perspectives of the experiments

The results of the Schibler team's research provide new research perspectives for cancer research and pharmaceutical development. By carrying out long-term observations of circadian rhythms in connection with medications, researchers can find out if specific preparations are more effective at certain times of day. Since defects in the cycle between protein synthesis and deactivation are associated with cancer, the circadian gene expression in the single cell also points out new directions for cancer research. The Leica AF6000 LX multidimensional fluorescence workstation is ideally suited to these and other future examinations of circadian rhythms in single cells.

> CB, VM

References

- [1] Balsalobre, A., F. Damiola, U. Schibler: A Serum Shock Induces Circadian Gene Expression in Mammalian Tissue Culture Cells. *Cell* 93: 929–937 (1998).
- [2] Nagoshi, E., C. Saini, C. Bauer, T. Laroche, F. Naef, U. Schibler: Circadian Gene Expression in Individual Fibroblasts. *Cell* 119, 693–705.

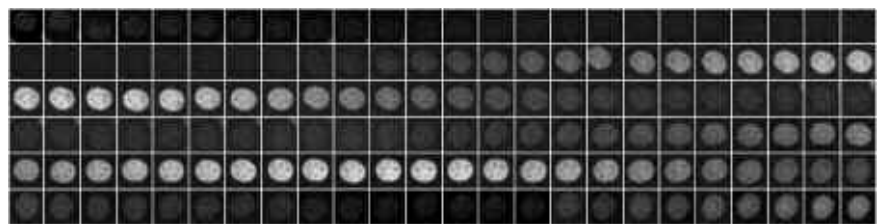


Fig. 3: Long-term observation of the Venus-tagged cell culture following shock treatment with greatly changed serum concentration. Images were captured every 30 minutes for three days.

This article was published in:

LaborPraxis, 4/2006
Vogel Industrie Medien
GmbH & Co. KG
Max-Planck-Strasse 7/9
D-97082 Würzburg
Germany

Contact:
Marc Platthaus
Phone +49 (0)931-418-2352,
Fax -2750
marc_platthaus
@laborpraxis.de
www.laborpraxis.de

Laser Microdissection in Plant Research

Fast, Powerful and Precise

An enormous challenge for plant researchers is the investigation of plant tissues at the molecular level. What are the processes of development and differentiation of plant tissue? How do plants develop disease resistance? How do they react to attacks from a virus, bacteria, fungi or insects? Answers to these questions will lead to a clearer picture of plant cell metabolism and help to better deal with plant diseases and thus increase biomass production.

DNA, RNA and protein analyses require highly pure cell populations where nucleic acids, antigens and cell structures remain intact for downstream experiments. Fresh tissue, cryo-fixed or paraffin embedded plant sections are examined histologically, cells are selected and then cut from the section using laser microdissection. This enables targeted enrichment of specific cell types, thereby increasing the selectivity of analytic and genetic investigations. Such targeted sample collection enables the gain of maximum information from minimum source material.

Plant research – the requirement for maximum flexibility

Initially, laser microdissection technology was developed for, and mainly used on, animal and human tissues. Recently, plant researchers started to use this highly precise tool for their own applications. The main barrier to overcome was sample preparation, particularly because the woody stems and thick cell walls are more difficult to cut than thinner, softer animal tissue and this is where advantage can be taken of the new Leica laser microdissection system – the Leica LMD6000, which comprises the Leica DM6000 (digital research microscope), new optics (optimized for laser microdissection), and powerful diode UV-

laser, ideal for tougher, thicker cells, such as plant cells! The Leica LMD6000 system is based on an upright microscope relying on gravity to drop the sample into a tube cap below, thus very large areas can be cut and dropped in one piece or very small cells can be dissected in the same way.

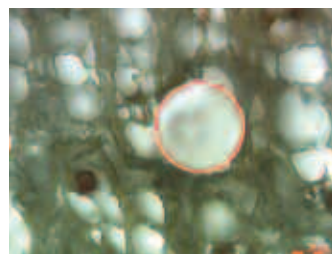
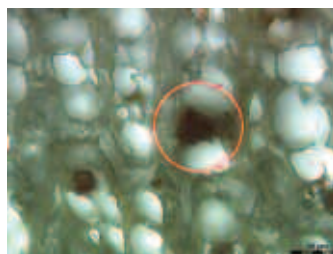
Can laser be used to cut a plant leaf?

A frequent request is to use the system for more demanding applications such as cutting leaves. This task is easy to perform with the Leica LMD6000, which makes it possible to microdissect fresh plant leaves in order to isolate defined tissue regions e.g. areas of variegation to analyse different compounds found within different pigments. These substances, in many cases, have not been investigated so far, because these compartments, for example, may only be approx. 200 µm diameter, and are too small for mechanical separation. In addition, one would need to collect several hundreds of these compartments to run NMR analysis. Now, using laser microdissection, direct and fast collection of tissue from the fresh material without former preparation for downstream analysis is possible.

Can the laser be used to cut wooden plant tissues?

Even wooden plant parts, like stem wood, from e.g. spruce can be easily cut with the Leica LMD6000 system. In order to isolate small areas containing storage substances separating the neighbouring cells, even 70 µm thick stem, longitudinal sections can be cut (Figures 1–3 and 4–7). For purer analysis thinner sections can be used because as the section becomes thicker the sample is less pure.

Fig. 1–3 (from left to right):
Laser Microdissection of small area of spruce section (73 µm thick):
– before microdissection
– after microdissection
– inspection mode



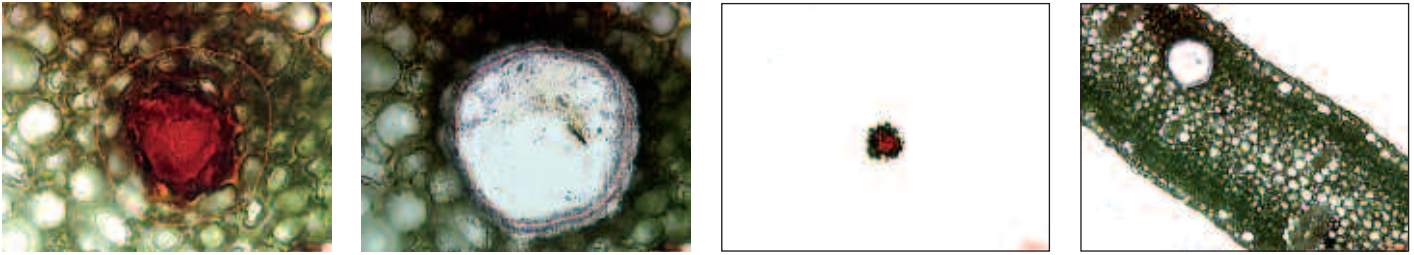


Fig. 4–7 (from left to right):
Laser Microdissection of oil vacuoles from a leaf cross-section:
– before microdissection
– after microdissection
– inspection mode (2 pictures)

What is the limitation on the size of area that can be cut?

In principle, areas of several millimetres can be collected directly in one piece into the PCR tube. Practically however, the shapes are drawn within the live image shown on the computer screen, which is approximately 3mm². It is also possible to draw larger areas beyond the entire screen (Figures 8–10). The shape of the dissectate is irrelevant, long tissue sections like stem can be isolated from the neighbouring cells (Figures 11–13). In addition, many separate areas can be cut into a single PCR tube thus increasing sample collection size.

High precision cutting

The Leica LMD6000 system is extremely flexible as it can also be used for the manipulation of micro organisms such as cyanobacteria (blue-green algae). Indeed, the laser can be used as a kind of scalpel to separate cyanobacterial chains. The growth media necessary for cell survival used to be the real challenge for laser cutting techniques. In the so called “sandwich procedure” the cyanobacteria are mount-

ed between two membrane slides. In this way the cells are held in liquid medium and laser beam can cut the sample with such a high level of accuracy that the bacteria are separated, without any damage to surrounding cells. As the sample drops into the tube cap, medium can be placed into the cap ready to receive the sample, reducing stress to delicate samples. Also fine plant structures such as root hairs or tobacco trichomes can be easily separated by laser beam (Figures 11–13).

Many innovative features of the Leica LMD6000 system such as the dedicated optics (at all magnifications), the powerful laser, fine laser beam adjustment and the gravity-collection result in maximum versatility of the system. Totally new approaches and investigations in numerous fields of Life Science are now available because development of this new microdissection platform has taken place in close co-operation with researchers.

> GZ

Acknowledgements

We thank the co-workers of the Max Planck Institute for Ecological Chemistry in Jena, Germany for their collaboration and providing the images: Dr. Bernd Schneider, Dr. Dirk Hölscher, Dr. Shenghong Li, Daniela Schmid and Dr. Susanne Textor.

This article was published in:

BIOspektrum 6/2006
Elsevier GmbH
Spektrum Akademischer Verlag
Slevogtstraße 3–5
D-69126 Heidelberg
Germany

Contact:
Dr. Britta Settmacher
Phone +49 (0)6221-9126-332, Fax -338
biospektrum@elsevier.com
www.biospektrum.de



Fig. 8–10 (from left to right):
Laser Microdissection of long, large areas in the range of mm² in a single piece from Arabidopsis stem-section (60 µm thick):
– before microdissection
– after microdissection
– inspection mode

Fig. 11–13 (from left to right):
Collection of tobacco trichome:
– before microdissection
– after microdissection
– inspection mode

True Confocal Microscopy

Affordable Excellence for Everyday Research

The application range in confocal microscopy has increased tremendously throughout the last decade: High quality fluorescent images have been an important key to new discoveries in biomedical research. Morphological studies, representing multiparameter fluorescence in fixed samples, are a major area of confocal microscopy. These applications require noise-free, high resolution imaging in order to see structural details, interconnections of organelles or proteins. A growing interest in confocal microscopy can be seen in clinical research, for example in pathology. High-resolution 3D confocal imaging may also give deeper insights and lead to new discoveries in pharmaceutical and biotechnological research.

Efficient and economic entry-level system with innovative concepts

In order to make confocal technology available to a wide range of users in their daily research, Leica Microsystems has developed a compact, easy to use and robust system in the entry-level class of confocal microscopes, the Leica TCS SPE. The new system covers the target applications in research and routine laboratory work. It requires minimal training effort and even confocal newcomers are able to quickly produce spectacular 3D images.

Despite its compactness the Leica TCS SPE confocal microscope features a range of innovative concepts and high quality standards without compromise in optical quality. The Leica TCS SPE is the only confocal system in its class with true spectral detection. It is based on a prism – spreading the light into its spectrum – and a detector device to select the spectral detection range. Thus, the system allows freely tunable spectral detection from 430 nm to 750 nm.

An Acousto Optical Tunable Filter (AOTF) with 0–100% excitation power minimizes exposure to light by individual tuning of the laser power for effective protection of fixed and living samples. A new optical concept, the Advanced Correction System (ACS), especially designed for the Leica TCS SPE allows perfect colocalization and ensures the high quality of the images.

Small but powerful – a highly integrated system

The Leica TCS SPE control box is no larger than a standard PC and contains up to four powerful solid state lasers: 488, 532 and 635 nm, the standard excitation lines for most common dyes (Figure 1), and a 405 nm laser, which is optionally available, for nuclear staining. These lasers have the advantage of being very durable and furthermore do not need any external cooling device.

The Leica TCS SPE has no specific room requirements. An anti-vibration table for the microscope and

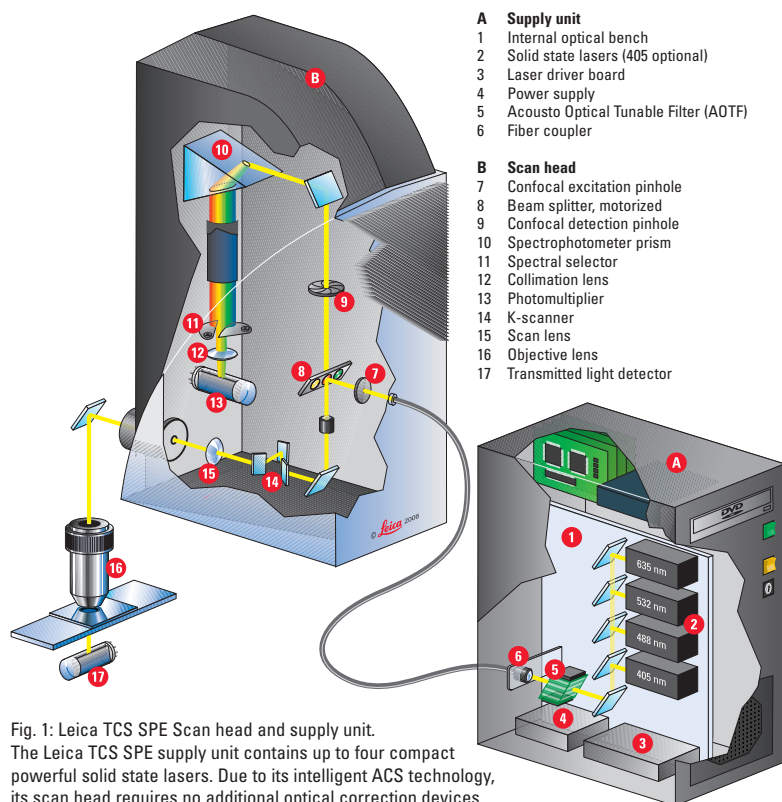


Fig. 1: Leica TCS SPE Scan head and supply unit. The Leica TCS SPE supply unit contains up to four compact powerful solid state lasers. Due to its intelligent ACS technology, its scan head requires no additional optical correction devices.

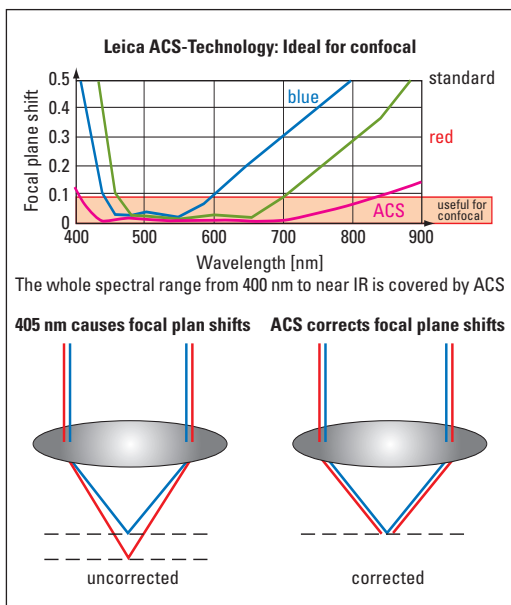


Fig. 2 : Leica ACS Technology

an ordinary workbench are fully adequate to arrange the system. Due to its small size and few requirements it surely fits in any laboratory. Good news for institutes and facilities where space is often rare and precious.

For high utilization of the system the control box contains an integrated PC with a DVD-writer, optimizing the system for multi-user environments, such as imaging facilities or shared resource laboratories. Individual user settings and results can be saved and reproduced later, while in the meantime other users may employ the system for other applications with their individual settings. The LAN connectivity of the Leica TCS SPE and USB sticks are additional possibilities of storing and transferring data.

New optics for perfect colocalization

Leica developed the new optical concept ACS for super positioning of all laser foci at only one point in the focal plane – from excitation to detection (Figure 2). In order to avoid two different optical section planes when working in the UV range, the UV laser is usually coupled in by a separate optical light path. However, optical correction devices are redundant with the new Leica Advanced Correction System. This correction concept is – like the true spectral detection – unique in this class of confocal microscopes. With the ACS the Leica TCS SPE achieves maximum transmission within the entire light band from 405 nm to infrared (Figure 3). As the system operates with only one AOTF and a shortened light path with single fiber coupling, it is a robust

and reliable confocal that requires minimum maintenance.

A new software platform for easy true confocal

An easy to use software interface inaugurates confocal microscopy to non-experienced users. With predefined system settings for defined dyes, confocal newcomers are able to acquire high quality images right from the start. The software is workflow-oriented and context sensitive to encourage error-free operation. On the other hand, the highly automated system is flexible for individual tuning and adapts to the needs of experienced users.

Users also benefit from the integrated software Leica Application Suite – Advanced Fluorescence (LAS AF), which operates throughout all advanced fluorescence systems from Leica: the high-end broadband confocal system Leica TCS SP5 as well as Leica’s widefield camera solutions AF6000 and AF6000 LX.

Tailored system solution for imaging facilities

With its small footprint, its long-life solid state lasers, the easy-to-use software interface for confocal newcomers and experts and the innovative ACS optics, the Leica TCS SPE fits perfectly into modern imaging facilities. Optimized for target applications like morphological studies and live cell imaging in small research groups as well as in multi-user environments, the Leica TCS SPE is therefore ideal for relieving the workload of high-end imaging systems.

> VM, PS

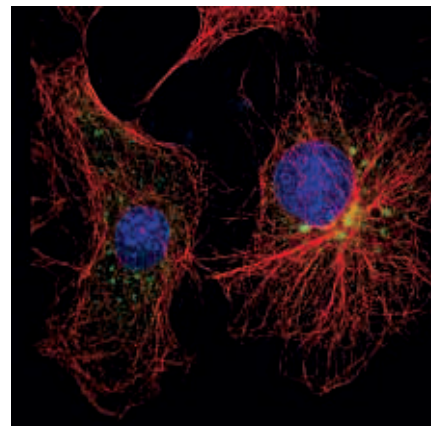


Fig. 3: COS cells
Green: uncharacterized protein;
Red: α -Tubulin, Cy3;
Blue: Nuclei, DAPI.
Courtesy of Prof. Wei Bian, Cell Research Center, Institute of Biochemistry and Cell Biology, SIBS, CAS, Shanghai, China

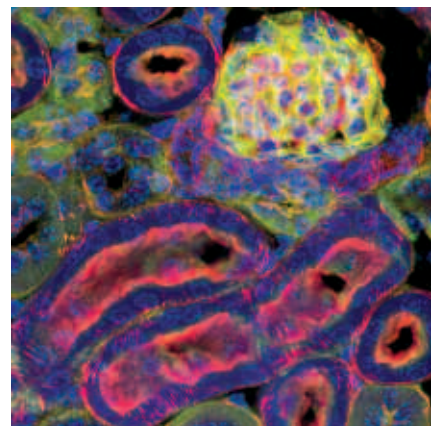


Fig. 4: Mouse kidney section
Green: glomeruli and convoluted tubules, Alexa 488 WGA;
Red: F-Actin (prevalent in glomeruli and brush border);
Blue: Nuclei, DAPI.

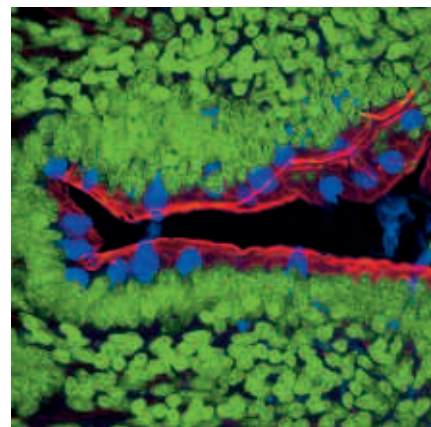


Fig. 5: Mouse Intestine Section
Green: Nuclei, Sytox, green;
Red: F-Actin (prevalent in brush border) Alexa 568;
Blue: mucus of goblet cells, Alexa 350 WG.

This article was published in:

G.I.T. Imaging & Microscopy 2/2006
GIT VERLAG GmbH & Co KG
Roesslerstrasse 90
D-64293 Darmstadt
Germany

Contact: Dr. Martin Friedrich
Phone: +49 (0)6151-8090-171, Fax: -176
m.friedrich@gitverlag.com
www.imaging-git.com/order/IMRegistration.pdf

Leica AM TIRF

The Way to Visualize Life's Secrets All Azimuths



TIRF (Total Internal Reflection Fluorescence) microscopy is the ideal technique for the study of cellular membranes and their environment. TIRF uses the evanescent field generated by total reflection to excite fluorophores. Instead of illuminating an entire specimen with excitation light, as in widefield fluorescence microscopy, the evanescent field only penetrates the specimen to a depth of 100–300 nm. Fluorochromes at deeper levels of the specimen are not excited. This method enables a substantially improved signal-to-noise ratio, providing the highest quality results for your examination.

With TIRF microscopy techniques, cells are not directly irradiated with laser light. The method of exciting fluorochromes via an evanescent field is particularly gentle and allows examination of live cell functions to be conducted over longer periods of time.

Leica Microsystems has developed a scanner technology for TIRF that greatly simplifies this otherwise very complicated technique. With the Leica AM TIRF, users can concentrate completely on their research work, while leaving instrument operation up to a powerful software program. Vesicle transport examina-

tions, analysis of interactions between molecules, applications in membrane research and even single molecule examinations can be tailored to specific needs using the Leica AM TIRF.

The software-controlled TIRF scanner automatically finds the correlation between the penetration depth of the evanescent field and the corresponding TIRF angles. With the Leica AM TIRF, complicated manual settings and complex calculations are unnecessary—the intelligent Leica AF6000 fluorescence software assumes these tasks, making the system convenient and easy to use.

Furthermore, various penetration depths can be reproduced at any time, and the laser beam can be rotated within the inlet pupil of the objective in order to generate various directions of the evanescent field.

This precise orientation of the laser beam is controlled through the Leica AF6000 software in a highly precise, yet easy-to-use way. This high level of interaction through the changing of the penetration depth and the direction of the evanescent wave, while maintaining homogeneity and image quality, reveals additional structural information that would otherwise remain hidden.

TIRF Membrane Research

Due to the excitation of fluorophores via an evanescent field, cells in the fluorescent light path are not directly irradiated with laser light. If an examination of membrane structures also requires quick switching of excitation wavelengths, Leica's new Fast Filter Wheels can be added to the widefield light path of the TIRF module; switching times of approximately 30–50 ms are easily achievable.

TIRF Single Molecule

With the Leica AM TIRF system it is easy to image single molecules close to the cell membrane. Typically, a powerful objective's resolving power in the z axis is

Fig. 1: Breast carcinoma tumour cells expressing a GFP tagged cell adhesion Molecule CD44 that is expressed on the cell membrane. A movie was generated showing very active membrane domains enriched for this protein at the surface in contact with the substrate, that were not seen at the surface beneath. These are better seen with the Leica AM TIRF instrument than with other observation techniques since no reflection is generated from the glass when imaging very near the coverslip surface. Courtesy of Dr. Maria C. Montoya, CNIO, Spanish National Cancer Center, Madrid.

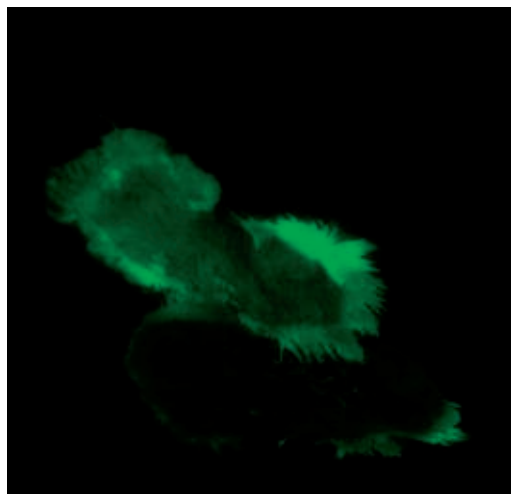




Fig. 2: Leica AM TIRF – innovative development in fluorescence simplifies complex microscopy method.

physically limited to approximately 200–300 nm by the wavelength of the light used. This physical restriction is eliminated by the high accuracy of the TIRF excitation of ~ 100 nm in the z axis.

TIRF Vesicle Transport

Due to strong background fluorescence, vesicle transport studies require an optimum signal-to-noise ratio. At the same time, stability for experiments lasting several hours or days is a must. Vesicle transport experiments are made simple with the Leica AM TIRF. With the addition of Leica’s special climate chamber to the TIRF system, temperature and CO₂ levels remain constant. The Leica AF6000 multidimensional fluorescence workstation with intelligent application software and hardware provides accurate results.

> DG

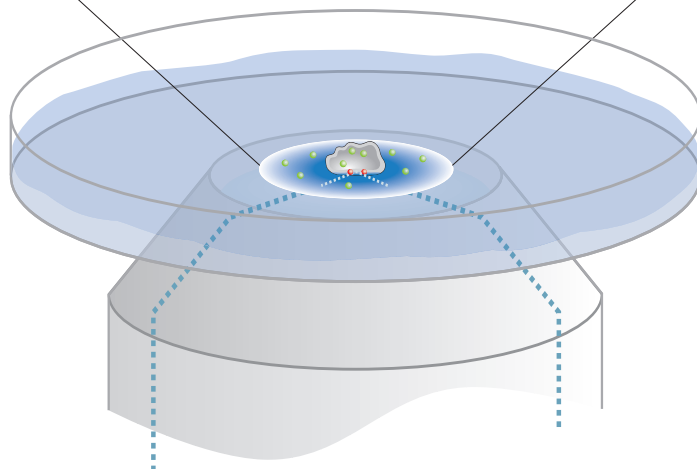
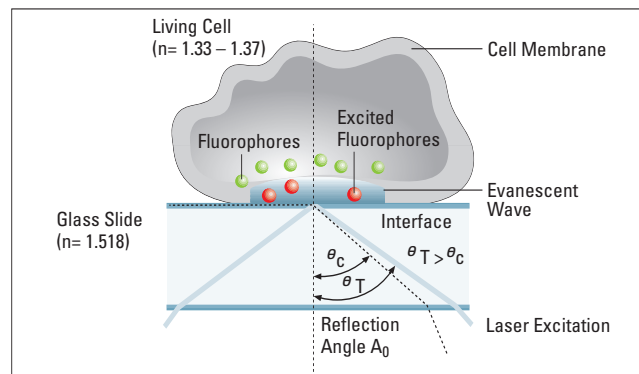


Fig. 3: Schematic view of TIRF illumination of live cells in a petri dish. The evanescent field is created when the laser beam is totally reflected at an interface; at an angle (θ_T) of more than θ_C . The evanescent wave’s intensity decreases exponentially depending on the wavelength, angle of incidence, and refractive index, and penetrates the specimen to an typical depth of 100–300 nm.

European Research Events

Here is just a sampling of some of the events Leica Microsystems will participate in or organise. For further information, please visit our website: www.leica-microsystems.com (click on events on the left).

2006

Presentación Leica LMD6000 – Nuevo Sistema de Micro-disección por Láser
October 25–26
Santiago de Compostela, Spain

Fraunhofer Life Science Symposium 2006
October 22–24
Leipzig, Germany

Het Instrument 2006
October 30 – November 3
Utrecht, Netherlands

Analisi in Vivo su singola cellula: I nuovi orizzonti della microscopia
November 6
Udine, Italy

IX Congresso Nazionale SIGU
November 8–11
Lido di Venezia, Italy

VII Curso Leica para el nuevo técnico-especialista en Microscopía Confocal Espectral
November 7–10
Bellaterra, Spain

Confocal User Club Meeting
November 15–17
Fiskebäckskil, Sweden

Medica 2006
November 15–18
Düsseldorf, Germany

Oxford University Workshop
November 29 – December 1
Oxford, UK

Les rencontres de l'INSERM 2006
December 12
Paris, France

2007

Congress of German society of Cellbiology
March, 14–17
Frankfurt, Germany

86th Annual Meeting of the German Society of Physiology
March 25–28
Hannover, Germany

31st Göttinger Neuroscience Conference
March 29 – April 1
Göttingen, Germany

FOM (Focus on Microscopy)
April 10–13
Valencia, Spain

7th ELMI meeting
April 17–20
York, UK

VIII Seminario di Microscopia Confocale
June
Rome, Italy

ELSO Meeting
September 1–9
Dresden, Germany

Contest

Win a Leica Digilux, Leica Wristwatch or other nice prizes!



Dear Reader:

Please give us your comments on this European Research Edition of reSOLUTION magazine. Send us your complete name and address of the Institute where you work, along with your comments by going to the following link by December, 2006:

www.leica-microsystems.com/EU-Research

Winners will be drawn in the first week of 2007 from all completed entries.



Manuel Beynon, Senior Sales Representative Research UK, presents a pair of Leica binoculars to the winner of the first contest, Dr. Keith Brain (left), Wellcome Trust Research Fellow (Dept. Pharmacology) & Research Fellow and Tutor (Keble College), University of Oxford.

Leica VT1200 and Leica VT1200 S

Good Vibrations for Great Sections in Neuroscience

“Ideally, a tissue slicer should generate large-amplitude and high-frequency movements of the cutting blade in a horizontal axis, with minimal vibrations in the vertical axis.”* (According to Prof. Peter Jonas, Institute of Physiology, University of Freiburg, Germany)

Leica Microsystems translated this into the semi automated Leica VT1200 and the semi- and fully automated Leica VT1200 S Vibrating Blade Microtome for cutting fresh and fixed tissues in neuroscience. Both instruments cut fresh and fixed tissue with minimal vertical deflection for sectioning results of highest quality with viable cells retained on the section surfaces. The vertical deflection can be measured by using the optional measurement device Vibrocheck and minimized below 1 µm by using an adjustment screw on the blade holder. The semiautomatic instrument is the preferred instrument for users who wish to manually select the desired section thickness prior to each section.

This fully automated instrument is recommended for multi user laboratories where users who prefer semi-

automated vibrating blade microtomes, and users who prefer fully automated instruments can work together. The mode of operation can be individually selected and up to eight individual parameter settings can be stored. The instrument was designed in collaboration with Prof. Dr. Peter Jonas and his group, Physiology Department Freiburg Germany.

> BK

* Reference

J. R. P. Geiger, J. Bischofberger, I. Vida, U. Fröbe, S. Pfitzinger, H. J. Weber, K. Haverkamp, P. Jonas: Patch-clamp recording in brain slices with improved slicer technology. *Pflügers Arch. – Eur. J. Physiol.* (2002) 443: 491–501.

Key features

- High section quality
- Vertical vibration measurement device “Vibrocheck”
- New blade holder for safe blade insertion
- Removable ice and buffer tray (drawer principle)
- Integrated palm rests
- Integrated LED illumination
- Optional magnifier
- Optional microscope
- External control panel



Imprint

reSOLUTION is the magazine for Leica Microsystems research customers

Publisher

Leica Microsystems GmbH, Wetzlar (Germany)

Editors

Didier Goor
Anja Schué

Editorial Address

Leica Microsystems GmbH
Ernst-Leitz-Straße 17–37
D-35578 Wetzlar (Germany)
www.leica-microsystems.com

E-Mail

Didier.Goor@leica-microsystems.com

Layout & Production

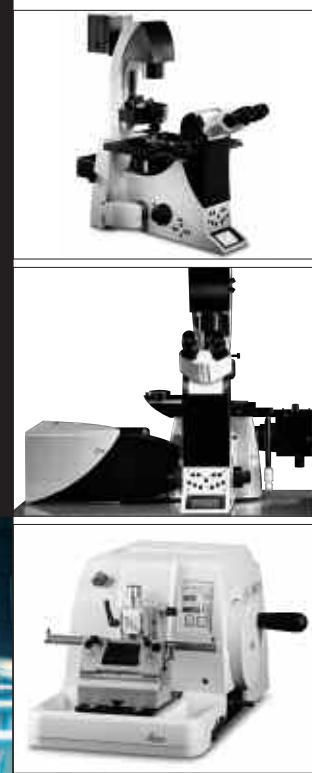
Central Marketing
Uwe Neumann

Cover Picture

Leica Microsystems GmbH
Fluorescence Applications

Contributing Editors

Christoph Bauer > CB
Alberto Diaspro > AD
Didier Goor > DG
Bianca Kircher > BK
Verena Mikeleit > VM
Anja Schué > AS
Peter Sendrowski > PS
Tanjef Szellas > TS
Thomas Zapf > TZ
Gerhard Zimmermann > GZ



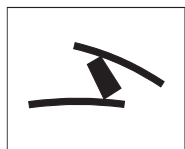
Leica & Science

Famous scientists working with instruments of Leica Microsystems

Leading scientists who use products of Leica Microsystems appreciate the superlative quality, technical perfection, technological standards, customer service and the wide choice.

When designing a new product, Leica Microsystems always puts the user first, offering the best possible solution for every application and every budget: from the routine microscope all the way through to high-end systems for life sciences and industry.

Winner 2005



Innovationspreis
der deutschen Wirtschaft
The World's First Innovation Award

www.leica-microsystems.com

Leica
MICROSYSTEMS